Protein-Protein Binding Kit of SUMO Conjugation Cascade by FRET Technology

David Bui
Richard Lauhead
Randall Mello
Michelle Tran

Bioengineering 175: Senior Design
University of California, Riverside
EXECUTIVE SUMMARY

The detection of protein-protein interactions is an important approach in many cellular processes. To meet market demands, a commercial-friendly kit must be designed to ensure cost-effective, easy to use, and produce efficient and reliable results. A number of existing methods such as the SPA kit and BRET2 kit have suggested that they are costly, relatively time consuming, and have limitations and concerns in the use of application. It is important to appreciate that the kit must be able to detect a wide array protein-protein interactions quickly and effectively which allow for the detailed study of the SUMOylation pathway. The goal of this proposal is to introduce the principal of SUMO Conjugation Pathway and FRET (Forster Resonance Energy Transfer) and to propose the methods of development of a FRET based kit to study protein-protein interactions, in particular SUMO1 and UBC9. Some methods of development include optimization of assay conditions, protein stabilities, and incorporation of secretion factors which comprise of positive and negative controls. By optimizing these conditions and parameters, we expect our in-vitro FRET-based assay kit will be commercially marketable and yield reproducible, quality results.

INTRODUCTION

BACKGROUND

SUMO (small ubiquitin-like modifier) belongs to a family of protein modifiers that changes the function, half-life, or location of proteins when it is covalently attached or detached from the target substrate. SUMO modifications play an important role in many biological pathways. Some of these pathways include regulatory gene expression and gene stability. The conjugation of SUMO to its particular substrates requires catalysis of several enzymes. These enzymes include E1 (sumo activating enzymes), E2(SUMO- conjugating enzymes), and E3 (SUMO Ligases). The specificity between SUMO, the catalyzing enzymes, and the substrate is not fully understood. There are several ways to currently detect protein-protein interactions. The most common method involves using an immunoblotting system. The immunoblotting system takes several days, and the number of compounds that can be screened is limited to the number of wells that can fit within a gel. Screening several hundred compounds with this method is tedious, slow, and costly. A FRET system requires a system of a Donor and Acceptor Fluorophores. This system lends itself to studying protein-protein interactions. Attaching a donor fluorophore to one protein and an acceptor fluorophore to another, the protein interaction can be determined by FRET occurring between the donor and acceptor. FRET allows for detection of protein-protein interactions without the use of immunoblotting. FRET relies on fluorescence, and fluorescence occurs within a frame of microseconds to seconds. So using a FRET system would allow for extremely fast screening of compounds very quickly. The number of FRET compounds is only limited by the number of wells within a plate.
PURPOSE OF THE PROJECT

The purpose of this project is to develop an in vitro high throughput FRET-based assay kit that will allow for the sensitive detection of a protein-protein interaction between SUMO and UBC9, the E2 enzyme. This kit will allow the user to screen compounds that could interfere with the conjugation of SUMO1 and UBC9. The kit will include all necessary proteins and solutions to run the assay. In the design of this kit, it will include development of a production procedure and optimization in each step to lessen production cost and time involved to produce the screening kit, in addition a production and kit manual will be included.

PREVIOUS WORK DONE BY OTHERS

*Scintillation Proximity Assay (SPA) kit by Perkin Elmer*

The SPA kit is designed for high-throughput screening (HTS) and relies on the principle of energy conversion of radioactive decay. The main component to the kit is the specially designed beads which contain a scintillant that emits light when excited by radio-labeled molecules. The energy conversion from radioactive decay to emitted photon can only occur when the bead is in close proximity to the radioactive molecule for example in the case of binding. The emitted photons are detected in the photomultiplier tubes of Scintillation counters for analysis. For the case of HTS, the SPA kit is available with Flashplates and Scintiplates in 96 and 384 well plates. The plates are designed to contain the target molecule conjugated with a scintillant bead in each well. The radio-labeled ligand designed for the sample molecule is added into the well with the sample molecule. Photons will be detected only when the combination sample-radio-ligand binds to the target conjugated scintillant bead. The disadvantages of the SPA kit are the cost of the scintillant beads as well as the radio-labeled ligands designed for each sample to be screened. Due to the nature of ligands being specific to individual molecules, using the SPA kit for High-Throughput Screening with large amounts of compounds would be time consuming and costly in production of ligands for each respectable compound. Radio-labeled ligands also have an additional disadvantage because of the radioactive beta-particles that are given off in the screening. This requires special screening parameters and can cause possible denaturation of proteins, altering the measured results.
The BRET\textsuperscript{2} kit is designed for G-protein coupled receptor (GPCR) high-throughput screening (HTS). The kit uses ß-Arrestin 2 conjugated with a Green Fluorescent Protein mutant (GFP) and a V2 Vasopressin receptor conjugated with the bioluminescent enzyme Renilla Luciferase (RLuc). When a substrate, a derivative of coelenterazine DeepBlueC, is added to the sample the RLuc enzyme catalyzes a reaction with DeepBlueC giving light as a byproduct. If, during the screening process a molecule causes a conformational binding change in the GPCR, The ß-Arrstestin2-GFP fusion protein comes in close contact with the V2-Vasopressin-Rluc-DeepBlueC fusion protein enzyme complex and the fluorescence energy of the enzyme complex is transferred to the GFP fusion protein and can be measured. The kit can also be designed to measure the absence of BRET in the screening for possible inhibitors to the GPCR. The kit comes with the plasmids coding the two fusion proteins required for BRET as well as the substrate DeepBlueC. One disadvantage of the BRET\textsuperscript{2} kit is that the kit is specifically designed for GPCR screening only and other protein-protein interactions cannot be observed and measured. Another disadvantage is that the kit requires the consumer to transform the plasmids into cells, express the protein, and purify the protein. This is time-consuming in the overall goal to measure protein-protein interactions in GPCRs in HTS Assays.

**PROJECT DESCRIPTION**

**PRIMARY OBJECTIVE**

To develop this kit, Assay conditions must be optimized. To do this, we will have to accomplish a series of tasks. We start with optimizing purification procedures, and for this process we will develop a protocol based off of a purification kit, and to determine the best process we will create 4 separate protocols. The first protocol will consist of using the minimum amount of materials required by the kit, the second protocol will have maximum values, and the third will be in between. Then after we determine purity we will run a fourth protocol that has an average of the two best protocols. Alongside that we will also be testing for sensitivity of the Flexstation 2 and its fluorescence readings, for this we will run a series of serial dilutions to determine the minimum amount of each protein that the flexstation can detect. Both of these two tasks we plan to finish in 4 weeks.

The next task is to lyophilize our protein to powder and when doing this we will either reduce the proteins completely to powder form, and also reduce it to a very highly concentrated solution then afterwards test for functionality. This is planned for 4 weeks to completion. After this the next step is to determine whether or not the purity of the protein will have a role in affecting the outcome of our screening assay, and for this we plan to introduce untagged proteins into our solution and see how it affects our FRET results and this will also take 4 weeks, but it will be done alongside the lyophilization.
Another step we plan to optimize will be the optimizing the expression of our proteins and try to determine the best conditions and the length of time for our proteins to be made, whether or not a 3 hour expression will be better than an overnight and figure out the best time in between. We plan for this task to take two weeks. And the other task we have planned to be run in conjunction to this will be testing for FRET sensitivity; for this task our goal here is to combine our two proteins and from there determine the minimum amount of our combined proteins that will give a FRET signal and then to determine the best concentrations of each.

The following two weeks we have to test for compound screening sensitivity and stability. For compound screening what our goal here is to be able to model our inhibitor mathematically, starting with the equation below, to determine the concentration required for the appropriate concentration of our proteins then afterwards we will actually test this and see if the lab results match with what we have expected.

\[
E + I + S \xrightleftharpoons[k_{-1}]{k_1} ES
\]

Our next objectives will be to test shelf life and then on to assembling the kit. For the shelf life we have planned a series of oxidation tests to see how long our products will last when open to air for prolonged periods of time and see if that has any affect on our proteins, and this is planned for 3 weeks. In this 3 week period we will also begin to put together our kit with all the components and the manual together and our primary goal should be reached.

**SECONDARY OBJECTIVE**

The secondary objective is to optimize production of materials for the previously mentioned kit, to make cost lower and also to incorporate positive and negative controls. To make the kit more cost and time efficient our goal is to put a secretion factor onto our genes so that as the proteins are produced it gets secreted outside the cell, whereas currently the proteins will be produced within the cell which then requires more time to purify. The purpose of including the positive and negative controls is to make our kit more complete so the consumers will not need to buy their own controls.
## METHODS

### PRIMARY GOAL

- **Protein Expression**
  - @ 25 deg for 16 hours, 25 deg for 3 hours, 37 deg for 3 hours

- **Fermentation**

- **Protein Purification**
  - Bradford, Protein Gel, Fluorescence Purity Check

- **Characterization**
  - Determine best purification method

- **Add UBC9**
  - to determine optimized amounts for inhibitors

- **Optimize FRET**
  - for SUMO1 and UBC9

- **Resuspend**
  - both powder and concentrate and test for function

- **Stability Testing**
  - Determine how long before oxidation occurs for tube

- **Design Kit and Manual**

- **Lyophilization**
  - To powder
  - To concentrate

### SUMO ASSAY KIT

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BUDGET

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CONCLUSION

To put it simply our aim for this project is to design a FRET based kit to study protein-protein interactions, more specifically the proteins SUMO1 and UBC9 in the SUMOylation pathway, and the overall goal is to design a high throughput screening kit for consumers also working with the SUMOylation pathway which allows them to screen many different types of compounds that would compete or interfere with SUMO binding to UBC9. A completed kit such as the one planned will save time for many researchers in that they will not need to produce the proteins themselves and if all the steps in the production can be optimized as we have planned it should also save them quite a bit of money.

What makes our product unique is that even though there are currently many different types of kits for different purposes there are only a few out there that are specifically geared towards protein-protein interactions such as the SPA kit, but as mentioned above the disadvantage to using the SPA kit is that to screen different samples there would be a need to design specific ligands for each case which would be far too time consuming. In the case of the BRET2 kit, it was designed for GPCR screening only, and so what makes our kit unique is the fact that researchers can screen for a wide array protein-protein interactions very quickly and efficiently using FRET which can then allow the study the SUMOylation pathway in more detail, the other current methods have problems in their specificity or time constraints. Currently the price has yet to be determined until completion of the optimization steps planned, but we are expecting something that would suit the market well, even though the number of facilities working on the SUMOylation pathway is fairly scarce.