# Using FRET-based Measurements of Protein Complexes to Determine Stoichiometry with the Job Plot

Francesca Mattiroli[1], Yajie Gu[1,2], and Karolin Luger[1]
[1]HHMI, University of Colorado Boulder, Dept. Chem. & Biochem., Boulder, CO, USA; [2]Colorado State University, Dept. Biochem. & Mol. Biol., Fort Collins, CO, USA

- **Determination of stoichiometry of protein-protein interactions is based on observed FRET maximum**
- **The CLARIOstar was used in a 384-well plate format to measure FRET and relevant controls for up to 4 interactions**
- **Determination of stoichiometry is assisted by a MARS data analysis template**

## Introduction

Measurement of stoichiometry is important for understanding biochemical reactions since these reactions are dependent on the interaction of at least 2 cellular components. Measuring these interactions has been limited to approaches that are useful for observing large molecular weight changes such as size-exclusion chromatography. Furthermore, these approaches require a large amount of purified cellular components.

With these limitations in mind we sought to provide a platform where protein-protein interactions could be observed. Herein we describe an adaptation of the Job plot[1]. Our approach uses a microplate reader, which was used to read FRET and relevant acceptor and donor control intensities. Using a microplate reader enables the use of small sample volumes (40 µl) in 384-well plates. Furthermore, 4 separate interactions can be studied on an individual plate.

## Assay Principle

A key factor of the Job plot, also called the continuous variation method, is that the total concentration of the 2 molecules is held constant[2]. It is the ratio of the 2 molecules that is changed. An observable parameter that is proportional to complex formation is employed, in this case FRET between the labelled proteins. Figure 1 depicts the expected assay results for a 1:1 protein-protein interaction.

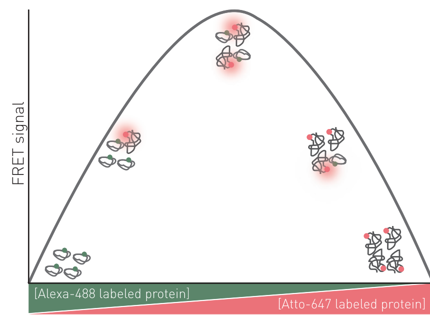


**Fig. 1:** Assay Principle for FRET Job plot

In the conditions where 100% donor labelled protein are present there is no FRET signal as the ratio shifts to larger amounts of acceptor the FRET signal increases until maximum FRET is obtained. Maximum FRET will indicate the stoichiometry of the interaction. Continued changes in the ratio toward greater acceptor will be associated with a decrease in FRET until zero FRET is again seen with 100% acceptor labelled protein.

## Materials & Methods

- Black 384-well microplates (Corning)
- BMG LABTECH CLARIOstar
- For a complete list of reagents and procedure please refer to Mattiroli et al.[1]

*Experimental Procedure*

Stock solutions with a 1 µM concentration were prepared for each protein, both labeled and unlabeled. Further dilution of these stocks were created such that diluted stocks have 2X concentration needed for the well reactions. Final well reactions are created by combining 20 µl each of the appropriate diluted stocks for proteins 1 and 2. This can be done according to the scheme depicted in Figure 2.

| | 1 | 2 | 3 | 4 | 5 | 6 | Protein 1 : Protein 2 ratio |
|---|---|---|---|---|---|---|---|
| C | B A | B A | B A | B A | B A | B A | Buffer control |
| D | S1 A 100 | S1 A 100 | S1 B 100 | S1 B 100 | S1 C 100 | S1 C 100 | 1:0 |
| E | S2 A 91.666 | S2 A 91.666 | S2 B 91.666 | S2 B 91.666 | S2 C 91.666 | S2 C 91.666 | 11:1 |
| F | S3 A 83.332 | S3 A 83.332 | S3 B 83.332 | S3 B 83.332 | S3 C 83.332 | S3 C 83.332 | 5:1 |
| G | S4 A 74.998 | S4 A 74.998 | S4 B 74.998 | S4 B 74.998 | S4 C 74.998 | S4 C 74.998 | 3:1 |
| H | S5 A 66.664 | S5 A 66.664 | S5 B 66.664 | S5 B 66.664 | S5 C 66.664 | S5 C 66.664 | 2:1 |
| I | S6 A 58.33 | S6 A 58.33 | S6 B 58.33 | S6 B 58.33 | S6 C 58.33 | S6 C 58.33 | 1.4:1 |
| J | S7 A 49.996 | S7 A 49.996 | S7 B 49.996 | S7 B 49.996 | S7 C 49.996 | S7 C 49.996 | 1:1 |
| K | S8 A 41.662 | S8 A 41.662 | S8 B 41.662 | S8 B 41.662 | S8 C 41.662 | S8 C 41.662 | 1:1.4 |
| L | S9 A 33.328 | S9 A 33.328 | S9 B 33.328 | S9 B 33.328 | S9 C 33.328 | S9 C 33.328 | 1:2 |
| M | S10 A 24.994 | S10 A 24.994 | S10 B 24.994 | S10 B 24.994 | S10 C 24.994 | S10 C 24.994 | 1:3 |
| N | S11 A 16.66 | S11 A 16.66 | S11 B 16.66 | S11 B 16.66 | S11 C 16.66 | S11 C 16.66 | 1:5 |
| O | S12 A 8.326 | S12 A 8.326 | S12 B 8.326 | S12 B 8.326 | S12 C 8.326 | S12 C 8.326 | 1:11 |
| P | S13 A 0.004 | S13 A 0.004 | S13 B 0.004 | S13 B 0.004 | S13 C 0.004 | S13 C 0.004 | 0:1 |
| | donor only control | | acceptor only control | | FRET experiment | | |

Protein 1 → Protein 2

**Fig. 2**: 384-well plate preparation layout

Rev. 09/2019
Keywords: **Stoichiometry, Job plot, Alexa488, AlexaFluor 647, Histone**

*Instrument settings*

Because of the multichromatic nature of the test performed appropriate setting of the gain for each chromatic was an important consideration. To measure the acceptor fluorescence gain was set using a sample with highest acceptor dye and no donor, for example well P3. Similarly, gain for donor fluorescence measurement was set on well with highest donor dye and no acceptor dye, such as well D1. For FRET measurement plate is first read and the well with max FRET signal is found. This well is used to perform gain adjustment.

| | | |
|---|---|---|
| Optic settings | Fluorescence, multichromatic, endpoint | |
| | Chromatic 1: Alexa 488 preset | |
| | LVF Ex | 488-14 |
| | Dichroic | Auto: 507.5 |
| | LVF Em | 535-30 |
| | Chromatic 2: Atto 647 preset | |
| | LVF Ex | 625-30 |
| | Dichroic | Auto: 647.5 |
| | LVF Em | 680-40 |
| | Chromatic 3: Alexa 488/Atto 647 FRET | |
| | LVF Ex | 488-15 |
| | Dichroic | Auto: 577.8 |
| | LVF Em | 680-40 |
| | Gain | Adjusted as described |
| General settings | Number of flashes | 50 |
| | Settling time | 0.1 s |

## Results & Discussion

As proof of principle we first looked at the interaction between histone binding protein and histone H3-H4. In the layout described in figure 2 histone binding protein is protein 1 and H3-H4 is protein 2. Figure 3 show the expected 1:1 binding interaction.

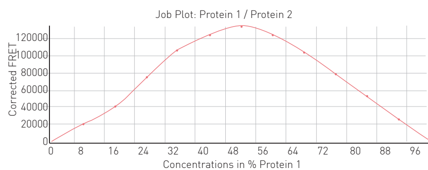


**Fig. 3:** Job plot for interaction between H3-H4 and histone binding protein

Figure 4 shows the results of 2 additional protein interaction tests. As you can see one of the interactions also exhibits a 1:1 stoichiometry while the other is an example of a 2:1 interaction stoichiometry.

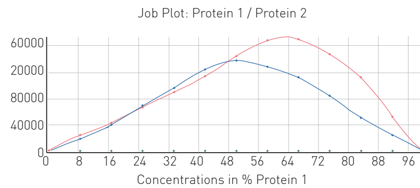


Fig. 4: Job plot depicting 1:1 and 2:1 protein-protein interaction stoichiometry

## Conclusion

The Job plot to assess protein-protein interaction stoichiometry has been successfully adapted to a microplate reader-based system. This enables both miniaturization to save on protein components and improved throughput, up to 4 protein pairs can be studied in one 384-well plate.

## References


1. Mattiroli, F. et al. FRET-based Stoichiometry Measurements of Protein Complexes *in vitro*. Bio. Protoc. (2018) 8: e2713. DOI: 10.21769/BioProtoc.2713

2. Huang, C.Y. Determination of Binding Stoichiometry by the Continuous Variation Method: The Job Plot. Methods Enzymol. (1982) 87: 509-525