

# Label-Free Quantification of Cooperative Protein-DNA Binding by Composition-Gradient Multi-Angle Light Scattering

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## Abstract

### Purpose

In vivo, biomolecular therapeutics may participate in complex interactions with their protein or DNA targets, resulting in binding stoichiometries other than 1:1, and the efficacy of these products may be limited by cooperativity, allosteric hindrance, or other mechanisms. Composition-gradient multi-angle light scattering (CG-MALS) enables the quantification of macromolecular associations without sample tagging, immobilization, or other modifications that could interfere with these phenomena. Here we measure the interaction between Cre recombinase and *loxP* DNA and observe changes in cooperativity and synopsis as a function of pH.

### Methods

Cre and *loxP* samples were prepared to stock concentrations in buffer and filtered to 0.02  $\mu\text{m}$ . Composition gradients were automated by the Calypso II hardware and delivered to downstream multi-angle light scattering and concentration detectors. Light scattering and composition data were fit to the appropriate model to determine stoichiometry and equilibrium dissociation constant ( $K_D$ ) at each binding site.

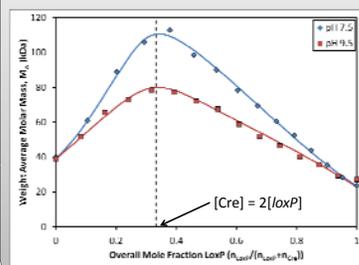
### Results

At pH 7.5 Cre binds *loxP* DNA with 2:1 stoichiometry, and the binding affinity of the second Cre protein to each palindromic *loxP* site is increased ten-fold, indicative of cooperative binding. This 2:1 complex self-assembles (synapsis) to form a final 4:2 stoichiometry. At pH 9.5, not only is synopsis abolished, but cooperativity is lost, and two Cre proteins bind each *loxP* with equivalent affinity.

### Conclusion

CG-MALS provides rapid quantification of the Cre-*loxP* interaction as a function of pH, elucidating the cooperativity in the protein-DNA binding as well as characterizing the self-assembly of Cre-*loxP* complexes to higher order structures. This simple, robust technique can be applied to a host of macromolecular interactions, making it ideal for the characterization of biomolecular interactions between drug products, their targets, host proteins involved in their clearance, and other interacting partners.

## Cre-*loxP* Interaction vs. pH

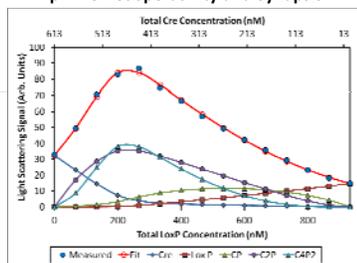


- The maximum molar mass occurs at the composition where  $[\text{Cre}] = 2/[\text{loxP}]$ . This indicates an overall 1:2 stoichiometric ratio.
- The molar mass measured at pH 7.5 is greater than the molar mass of a  $(\text{Cre})_2/(\text{loxP})$  complex, as expected when  $(\text{Cre})_4/(\text{loxP})_2$  is formed.

## Results

### Best Fit Analysis as a Function of pH:

#### pH 7.5: Cooperativity and Synopsis



#### Cre-*loxP* interaction includes synopsis.

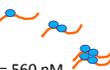
The data must be described by model that includes three complexes— $(\text{Cre})(\text{loxP})$ ,  $(\text{Cre})_2/(\text{loxP})$ , and  $(\text{Cre})_4/(\text{loxP})_2$

#### Cre-*loxP* interaction is cooperative.

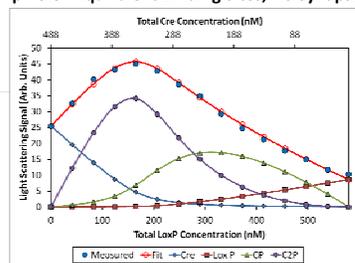
First binding event,  $K_D = 100$  nM

Second binding event,  $K_D = 10$  nM

$(\text{Cre})_2/(\text{loxP})$  dimerizes (synapsis) with  $K_D = 560$  nM



#### pH 9.5: Equivalent Binding Sites, No Synopsis



#### Synapsis is lost.

The measured LS data is consistent with  $(\text{Cre})_2/(\text{loxP})$  formation and does not allow for  $(\text{Cre})_4/(\text{loxP})_2$

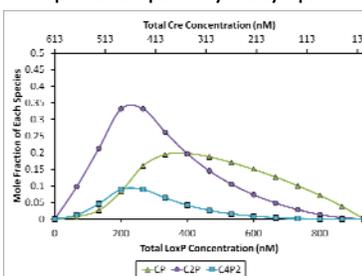
#### Cre binds *loxP* with equivalent affinity at each site.

Binding site affinity,  $K_D = 24$  nM



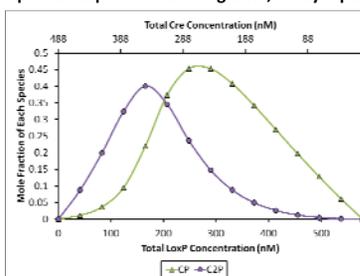
### Distribution of Species as a Function of pH:

#### pH 7.5: Cooperativity and Synopsis



The fraction of  $(\text{Cre})_4/(\text{loxP})_2$  complex ( $\blacktriangle$ ) reaches a maximum the total Cre concentration equals the total *loxP* concentration. Both the  $(\text{Cre})_2/(\text{loxP})$  complex ( $\bullet$ ) and synapse tetramer,  $(\text{Cre})_4/(\text{loxP})_2$  ( $\blacktriangle$ ), reach a maximum where the overall concentration of Cre is twice the overall concentration of *loxP*. The fractions of Cre and *loxP* monomers have been left off the graph for clarity.

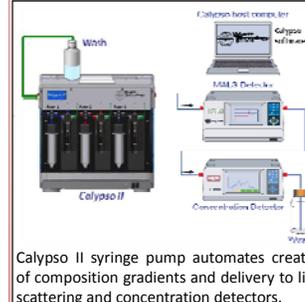
#### pH 9.5: Equivalent Binding Sites, No Synopsis



The fraction of  $(\text{Cre})_2/(\text{loxP})$  complex ( $\blacktriangle$ ) reaches a maximum at the composition  $[\text{Cre}]_{\text{total}} = [\text{loxP}]_{\text{total}}$  and the  $(\text{Cre})_2/(\text{loxP})$  complex ( $\bullet$ ) reaches a maximum at the composition  $[\text{Cre}]_{\text{total}} = 2/[\text{loxP}]_{\text{total}}$ . Since cooperativity is lost, there is significantly more of the  $(\text{Cre})_2/(\text{loxP})$  species than at pH 7.5. The fractions of Cre and *loxP* monomers have been left off the graph for clarity.

## Methods

### Composition-Gradient Multi-Angle Light Scattering:



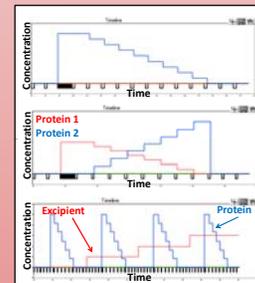
Calypso II syringe pump automates creation of composition gradients and delivery to light scattering and concentration detectors.

Analysis of CG-MALS data provides

- Self- and hetero-association, affinity and stoichiometry
- Nonspecific interactions, both attractive and repulsive
- Reversible and irreversible kinetics of aggregation and dissociation

### Typical CG-MALS Method Design:

- Single species gradient
  - Molecular weight
  - Self-virial coefficient
  - Self-association (affinity and stoichiometry)
- Two-species crossover gradient
  - Hetero-association
  - Cross-virial coefficient
- Titration
  - Buffer and excipient effects
  - Species with large molecular weight differences



## Conclusions

At pH 7.5, the CG-MALS data measured for the Cre-*loxP* interaction can only be described by a model that includes Cre-*loxP* interactions  $>2:1$ . The formation of the  $(\text{Cre})_2/(\text{loxP})$  complex exhibits cooperative binding with the  $K_D$  at the second binding site decreasing by 10x after binding of the first Cre to *loxP*. The  $(\text{Cre})_2/(\text{loxP})$  complex further dimerizes with affinity  $K_D \sim 560$  nM.

At pH 9.5, the CG-MALS data are consistent with significantly less complex formation than at pH 7.5. Here, the best fit includes a  $(\text{Cre})_2/(\text{loxP})$  interaction in which cooperativity has been lost, and the two Cre binding sites are equivalent with  $K_D = 24$  nM. This results in a greater concentration of 1:1  $(\text{Cre})_2/(\text{loxP})$  complex compared to the interaction at pH 7.5.

