Supporting information for Bennett *et al*. (2002) *Proc. Natl. Acad. Sci. USA,* 10.1073/pnas.182393899.

Equilibrium Analysis of Trivalent Analyte Data

The bivalent analyte model as previously described (1, 2) derives K_{D1} and K_{D2} for the sequential binding reactions that produce single- and double-liganded analyte molecules. We extended this approach to develop a trivalent analyte model that accounts for the possibility of triple-liganded molecules by deriving the additional equilibrium constant K_{D3}. The sequential reactions are as follows, in which A is the ligand and B is the trivalent analyte:

$$A + B \underset{kd1}{\overset{ka1}{\rightleftharpoons}} A \bullet B \qquad K_{D1} = \frac{k_{d1}}{k_{a1}} = \frac{[A][B]}{[A \bullet B]} \qquad \textbf{[1]}$$

$$A + A \bullet B \underset{kd2}{\overset{ka2}{\rightleftharpoons}} A_2 \bullet B \qquad K_{D2} = \frac{k_{d2}}{k_{a2}} = \frac{[A][A \bullet B]}{[A_2 \bullet B]} \qquad \textbf{[2]}$$

$$A + A_2 \bullet B \underset{kd3}{\overset{ka3}{\rightleftharpoons}} A_3 \bullet B \qquad K_{D3} = \frac{k_{d3}}{k_{a3}} = \frac{[A][A_2 \bullet B]}{[A_3 \bullet B]} \qquad \textbf{[3]}$$

The total ligand concentration, A_{tot}, is given by the expression

$$[A_{tot}] = [A] + [A \bullet B] + 2[A_2 \bullet B] + 3[A_3 \bullet B]. \qquad \textbf{[4]}$$

The equilibrium response for a trivalent analyte is proportional to the sum of the concentrations of single-, double-, and triple-liganded analyte,

$$R_{eq} \propto [A \bullet B] + [A_2 \bullet B] + [A_3 \bullet B], \qquad \textbf{[5]}$$

which can be rearranged by substituting Eqs. **1-4**:

$$R_{eq} \propto \frac{[A_{tot}]}{3} + \left(\frac{2[B]}{3K_{D1}} - \frac{1}{3} \right)[A] + \left(\frac{[B]}{3K_{D1}K_{D2}} \right)[A]^2 \qquad \textbf{[6]}$$

The concentration of free ligand, [*A*], is derived by solving a cubic equation based on Eqs. **1-4**. Substituting [*A*] into Eq. **6** yields the trivalent analyte equation:

R_{eq}=Scale*(A_{tot}+((-1/3+(2*B)/(3*KD1))*(-4*KD3+(2*2^(1/3)*KD3*
(-9*B*KD2-9*KD1*KD2+4*B*KD3))/
(2*B^3*(27*KD2-8*KD3)*KD3^2+27*B^2*KD1*KD2*KD3*(2*KD3+9*A_{tot})+
sqrt(B^3*KD3^2*(4*KD3*(9*B*KD2+9*KD1*KD2-4*B*KD3)^3+
B*(2*B*(27*KD2-8*KD3)*KD3+27*KD1*KD2*(2*KD3+9*A_{tot}))^2)))^(1/3)+
(2^(2/3)*(2*B^3*(27*KD2-8*KD3)*KD3^2+27*B^2*KD1*KD2*KD3*(2*KD3+9*A_{tot})+
sqrt(B^3*KD3^2*(4*KD3*(9*B*KD2+9*KD1*KD2-4*B*KD3)^3+
B*(2*B*(27*KD2-8*KD3)*KD3+27*KD1*KD2*(2*KD3+9*A_{tot}))^2)))^(1/3))/
B))/6+
(B*(-4*KD3+(2*2^(1/3)*KD3*(-9*B*KD2-9*KD1*KD2+4*B*KD3))/
(2*B^3*(27*KD2-8*KD3)*KD3^2+27*B^2*KD1*KD2*KD3*(2*KD3+9*A_{tot})+
sqrt(B^3*KD3^2*(4*KD3*(9*B*KD2+9*KD1*KD2-4*B*KD3)^3+
B*(2*B*(27*KD2-8*KD3)*KD3+27*KD1*KD2*(2*KD3+9*A_{tot}))^2)))^(1/3)+
(2^(2/3)*(2*B^3*(27*KD2-8*KD3)*KD3^2+27*B^2*KD1*KD2*KD3*(2*KD3+9*A_{tot})+
sqrt(B^3*KD3^2*(4*KD3*(9*B*KD2+9*KD1*KD2-4*B*KD3)^3+
B*(2*B*(27*KD2-8*KD3)*KD3+27*KD1*KD2*(2*KD3+9*A_{tot}))^2)))^(1/3))
/B)^2)/(324*KD1*KD2))/3

R_{eq} = measured equilibrium response in resonance units (RU).

A_{tot} = total concentration of ligand (M) (proportional to maximum response, R_{max}).

B = concentration of free analyte (M).

Scale = conversion factor (RU/M) =100 × M_r(analyte) or ~2.6 × 10^7 for HD exon 1.

KD1, KD2, KD3 = affinity constants.

1. West, A. P. & Bjorkman, P. J. (2000) *Biochemistry* **39**, 9698–9708.

2. West, A. P., Giannetti, A. M., Herr, A. B., Bennett, M. J., Nangiana, J. S., Pierce, J. R., Weiner, L. P., Snow, P. M. & Bjorkman, P. J. (2001) *J. Mol. Biol.* **313**, 385–397.