

HLA-B27 Binding Assay

jlp 6/8/95

- 1) As of today the B27 protein concentration = 9 mg/mL
- 2) Dilute the protein 2-fold with 1xPBS:
 - 75 μ l B27 (9 mg/mL)
 - 75 μ l 1xPBS
 - 2 μ l 0.1M DTT
- 3) Incubate on ice for 5 minutes.
- 4) Meanwhile, prepare the spin columns (Biospin 6 and Biospin 30 from Biorad; they are stored in the cold room).

Biospin -6 column equilibration:

- a) Cut the bottom tip off the column
- b) Let it completely drain
- c) Add 2 ml of **borate buffer**, pH 10.4
- d) spin @ 1500 RPM for 5 minutes
- e) Repeat steps c and d

Biospin-30 column equilibration:

- a) Cut the bottom tip off the column
- b) Let it completely drain
- c) Add 2 ml of **1 X PBS** buffer, pH 10.4
- d) spin @ 1500 RPM for 5 minutes
- e) Repeat steps c and d

Finally, spin all the columns @ 1500 RPM for 1 minute.

- 5) After the 5-minute incubation, add 10 μ l of 4M K₂HPO₄-KOH, pH 12.0 and incubate on ice an additional 5 minutes.
- 6) This preparation is ~160 μ l; split it into two Biospin-6 columns (i.e. load 80 μ l per column) and centrifuge @ 1500 RPM for 5 minutes.
- 7) Take the flow through and load it into Biospin-30 columns and spin for 5 minutes @ 1500 RPM.
- 8) Pool all your fractions, and dilute them with 1xPBS to get the necessary volume for all your assays (i.e. you need 25 μ l of B27 per assay; if you are doing 45 assays then you will need aprox. 1.2 mL) and read the A₂₈₀.

Note: for the sake of reproducibility it is best when the A_{280} is between 0.67 and 1.00.

NoteII: It is good enough to do the assays in duplicate because the results are quite reproducible.

- 9) The batch of ^3H labeled peptide is around $51 \mu\text{M}$, but check this concentration before actually using it. To do this just:
- take a $100\mu\text{l}$ aliquot of the labeled peptide and put it into the microcuvette.
 - Read the absorbance spectrum from 200-300 nanometers.
 - Determine the A_{274} and divide it by 1400 to get the molar concentration of peptide.

Once you have determined the concentration of tritium-labeled peptide, dilute it with 1xPBS to a final concentration of $4 \mu\text{M}$.

10) Actual binding Assay:

Mix the following:

16 μl of $4 \mu\text{M}$ labeled peptide
40 μl of the desired [] of inhibitor peptide***
25 μl of the empty B27 protein

Note: The B27 is the last thing you add!

Incubate O/N on a shaker or mixer @ R.T.

***The first time the binding assay is done, start with 5 dilutions ranging from $0.6 \mu\text{M}$ to $10.0 \mu\text{M}$. If this is not good, then try $2.5 \mu\text{M}$ to $40 \mu\text{M}$. Dilute the peptide with 1xPBS, pH 7.0.

Control: a triplicate of an assay with labeled peptide only (no unlabeled peptide in it).

- 11) The next day take your samples out and purify each of them through a Biospin-30 column (don't equilibrate the columns).
- 12) Count the samples on the scintillation counter and calculate the K_D 's.