

HLA-B27 Binding Assay

1) As of today the B27 protein concentration = 9 mg/mL

2) Dilute the protein 2-fold with 1xPBS:

\[
\begin{align*}
75 \mu l & \text{ B27 (9 mg/mL)} \\
75 \mu l & \text{ 1xPBS} \\
2 \mu l & \text{ 0.1M DTT}
\end{align*}
\]

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 10µl of 4M K₂HPO₄-KOH, pH 12.0 and incubate on ice an additional 5 minutes.

6) This preparation is ~160µl; split it into two Biospin-6 columns (i.e. load 80µl 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 25µl 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.

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

9) The batch of $^3$H labeled peptide is around 51 µM, but check this concentration before actually using it. To do this just:

a) take a 100µl aliquot of the labeled peptide and put it into the microcuvette.
b) Read the absorbance spectrum from 200-300 nanometers.
c) 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 µM.

10) Actual binding Assay:

Mix the following:

16 µl of 4 µM 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 µM to 10.0 µM. If this is not good, then try 2.5 µM to 40 µM. 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.