Alpha protein-protein interaction: Competition binding curve experiment

1. Goal

- Create a competition curve to determine Kd in Alpha assay

2. Reagents

Component	Vendor	Catalog number
EGFR-Fc	R&D Systems	#344-ER
Biotin-EGF	Invitrogen	#E-3477
Unlabeled EGF	Invitrogen	#E-3476
Streptavidin Donor beads	PerkinElmer	#6760002S
Protein A AlphaLISA Acceptor beads	PerkinElmer	#AL101C
96-well ½ AreaPlate	PerkinElmer	#6005560
Assay buffer : PBS + 0.5% BSA	In-house	

3. Assay principle

This assay is designed to examine the interaction of epidermal growth factor (EGF, "the ligand") with its cognate receptor, epidermal growth factor receptor (EGFR, "the receptor"). EGF is a 53 amino acid small protein. Its discovery won Stanley Cohen the Nobel Prize in Medicine in 1986. EGFR is a receptor tyrosine kinase that is located on the cell surface. When EGF binds, it activates the tyrosine kinase activity of EGFR which begins a signaling cascade in the cell. This activity is frequently aberrant in cancer and inhibiting the EGFR response is an active area of research. This research field has spawned several clinical drugs including Tarceva (Genentech), Erbitux (ImClone/BMS), and Vectibix (Amgen).

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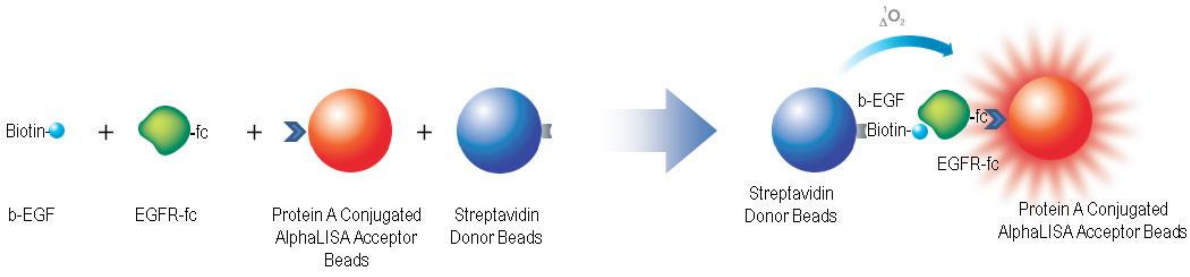

Figure 1. Alpha protein-protein assay design.

4. Reagent preparation

4.1 Preparation of EGFR-Fc (50 µg, MW=95.1 kDa):

a. Reconstitute EGFR-Fc in 0.5 mL PBS, to obtain 0.1 mg/mL (=100 µg/mL = 1.05 µM EGFR-Fc)
b. Dilute 1:100 in PBS + 0.5% BSA (10 µL EGFR-Fc + 990 µL [PBS + 0.5% BSA]) to get a 10.5 nM solution
c. Prepare 0.5 nM stock solution:

	[Final]	[Intermediate]	Vol of dilution	Buffer (PBS + 0.5% BSA)
EGFR-Fc	0.1 nM	0.5 nM	70 µL of 10.5 nM EGFR-Fc	1400 µL

4.2 Preparation of Biotin-EGF (20 µg, MW~6300):

a. Reconstitute Biotin-EGF in 0.5 mL deionized water to obtain 40 µg/mL = 6.35 µM
b. Prepare a 1:100 dilution in [PBS + 0.5% BSA] (10 µL biotin-EGF + 990 µL PBS + 0.5% BSA) to get a 63.5 nM solution
c. Prepare 5 nM stock solution:

	[Final]	[Intermediate]	Vol of dilution	Buffer (PBS + 0.5% BSA)
Biotin-EGF	1 nM	5 nM	100 µL of 63.5 nM biotin-EGF	1170 µL

4.3 Preparation of unlabeled EGF (100 µg, MW~6045):

d. Reconstitute Biotin-EGF in 0.5 mL deionized water to obtain 200 µg/mL = 33 µM
e. Prepare 1:3 dilutions in [PBS + 0.5% BSA]:

Dilution	[Final] (M)	[Intermediate] (M)	Vol of dilution	Buffer (PBS + 0.5% BSA)
1	1 µM	5 µM	100 µL of 33 µM stock	560 µL
2	333 nM	1666.7 nM	200 µL of dilution 1	400 µL
3	111 nM	555 nM	200 µL of dilution 2	400 µL
4	37.03 nM	185 nM	200 µL of dilution 3	400 µL
5	12.34 nM	61.7 nM	200 µL of dilution 4	400 µL
6	4.12 nM	20.6 nM	200 µL of dilution 5	400 µL
7	1.37 nM	6.86 nM	200 µL of dilution 6	400 µL
8	0.45 nM	2.28 nM	200 µL of dilution 7	400 µL
9	0.15 nM	0.76 nM	200 µL of dilution 8	400 µL
10	0.05 nM	0.25 nM	200 µL of dilution 9	400 µL
11	0.017 nM	0.085 nM	200 µL of dilution 10	400 µL
12	0 nM	0 nM	0	500 µL

5. Prepare 5x working solution (100 µg/mL) of Protein A AlphaLISA Acceptor beads:

25 µL Acceptor beads (5 mg/mL) + 1225 µL buffer (PBS + 0.5% BSA)

6. (During 2nd incubation): Prepare 5x working solution (100 µg/mL) of Alpha Streptavidin Donor beads:

25 µL Donor beads (5 mg/mL) + 1225 µL buffer (PBS + 0.5% BSA)

7. Assay protocol for a 96-well ½ AreaPlate (Total assay volume of 50 µL)

Refer to plate map in section 8, on next page. You can use a multi-channel repeat pipettor to quickly dispense reagents into the plate.

Protein-protein interaction assay
1. Add 10 µL biotin-EGF
2. Add 10 µL unlabeled EGF (dilution series)
3. Add 10 µL EGFR-Fc
4. Incubate 60 min at room temperature
5. Add 10 µL Protein A Acceptor beads (final conc. 20 µg/mL)
6. Incubate 60 min at room temperature
7. Add 10 µL Streptavidin Donor beads (final conc. 20 µg/mL)
8. Incubate 30 min at room temperature
9. Read on an EnVision or EnSpire

8. Map for 96-well ½ AreaPlate (Samples in triplicate)

	1 μM unlabeled EGF	333 nM unlabeled EGF	111 nM unlabeled EGF	37.0 nM unlabeled EGF	12.34 nM unlabeled EGF	4.11 nM unlabeled EGF	1.37 nM unlabeled EGF	0.46 nM unlabeled EGF	0.15 nM unlabeled EGF	0.05 nM unlabeled EGF	0.017 nM unlabeled EGF	0 nM unlabeled EGF
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A 0.1 nM EGFR-Fc, 1 nM biotin-EGF												
B 0.1 nM EGFR-Fc, 1 nM biotin-EGF												
C 0.1 nM EGFR-Fc, 1 nM biotin-EGF												
D												
E												
F												
G												
H												