NPY ASSAY

NPY PURIFICATION:

Preparation of Reagents:

Solutions:

Acetonitrile (100%)

Buffer A: 1% TFA in 18 MΩ H_2O
(Add 10 mL TFA to 990 mL 18 MΩ H_2O)

Buffer B: 60% acetonitrile and 1% TFA in 18 MΩ H_2O
(Add 120 mL acetonitrile and 2 mL TFA to 78 mL 18 MΩ H_2O)

Columns:

Peninsula Laboratories Inc
Cat # Y-1000; SEP Columns packed with C18 sorbent

Test tubes:

FisherBrand 12 x 75 mm polystyrene test tubes
Cat # 14-961-10A

Procedure:

1) Set up columns and test tubes in racks. Be sure to include a column for a standard NPY concentration. Do not place columns above tubes until eluting the sample with buffer B.
2) Prepare standard NPY solution (~2 - 5 ng/mL recommended) in same buffer as samples were prepared. (This can be done using the "Standard Serial Dilutions" from the NPY ELISA protocol.)
3) Wash columns with 100% acetonitrile. Be sure all solution is down to column sorbent prior to the next step.
4) Add 3 mL Buffer A. Repeat two more times. After 3rd Buffer A wash, be sure all solution is down to the column sorbent before next step.
5) Load samples onto columns. Be sure all solution is down to column sorbent prior to proceeding with Buffer A washes.
6) Run 3 mL Buffer A through column. Repeat two more times. After 3rd Buffer A wash, be sure all solution is down to column sorbent.
7) Place columns over test tubes.
8) Add 3 mL Buffer B to elute NPY sample. This should be collected in the test tubes.
9) After all solution has been collected in the tubes, dry the samples in a speed vac overnight. *[If planning on running NPY ELISA the next day, be sure to complete NPY ELISA protocol up through step 4.]*

NPY ELISA

Preparation of Reagents:

Buffers: (store at 4°C)

Assay Buffer:
1.42 g Na_2HPO_4 (dibasic)
0.2 g KH_2PO_4
1 mL Tween 20
Bring to 1 L, pH 7.4
(500 mg BSA + 100 mL buffer made day of use)

Wash Buffer:
1.42 g Na_2HPO_4 (dibasic)
0.2 g KH_2PO_4
1 mL Tween 20
Bring to 1 L, pH 7.4

Coating Buffer:
4.3 g $NaHCO_3$
5.3 g Na_2CO_3
Bring to 1 L, pH 9.4

Blocking Buffer:
8.0 g NaCl
1.42 g NaH_2PO_4 (monobasic)
0.2 g KH_2PO_4
0.2 g KCl
Bring to 1 L, pH 7.4
(250 mg BSA + 50 mL buffer made day of use)

Plate: Corning Costar (Cat #3361) EIA/RIA plate

NPY antibody: Rabbit Anti-NPY, Bachem Cat #T-4070
Do NOT follow reconstitution directions on data sheet. Reconstitute the 50 uL lyophilized serum in 500 uL 18 MΩ H_2O. Separate into 10 uL aliquots and store at -20°C.

Biotinylated NPY (Bio-NPY): Bachem Cat #H-5674
Reconstitute 0.5 mg in 500 uL 18 MΩ H_2O (1 mg/mL). Store in 10 uL aliquots as Bio-NPY Stock solution at -20°C.

Streptavidin-HRP (SA-HRP): BD Pharmingen Cat #554066
Store at 4°C.

TMB: 1 Step Ultra TMB-ELISA, Pierce Cat #34028
Store at 4°C.

2N H_2SO_4

NPY Standards: Bachem Cat #H-6375
Reconstitute to 1 mg/mL with 18 MΩ H_2O. Store at 4°C.

Procedure:

Preparations to be done the day prior to the experiment:

1) Make all buffers as discussed above and store at 4°C. Prepare all lyophilized reagents as above, if haven't already done so.
2) Make coating buffer/anti-NPY antibody solution (1:25,000 dilution): add 10 uL anti-NPY antibody (aliquots stored at -20°C) to 24.99 mL of coating buffer in 50 mL conical tube. Vortex.
3) Obtain 96 well EIA/RIA plate
4) Add 100 uL of coating buffer/anti-NPY antibody solution to each well. Cover plate with plastic cover and incubate <u>overnight</u> at 4°C.

Day of Experiment

5) Fill out a plate map, leaving 2 wells for non-specific binding (NSB) and 2 wells for total binding (TB). No blank necessary.
6) Mix 500 mg BSA with assay buffer to 100 mL total. This is the working assay buffer to be used for making standards and throughout the remainder of the experiment.
7) Reconstitute NPY samples in 200 uL assay buffer. Vortex.
8) Remove plate from refrigerator and wash 1X with 300 uL/well of wash buffer.
9) Mix 250 mg BSA with blocking buffer to 50 mL total. Add 300 uL to each well and incubate for 1 hour.
10) During incubation with blocking buffer, make the standards as explained in the table below. Also make any sample dilutions with assay buffer during this time.

    Start with NPY Standard stock solution (4°C). Make standards as follows:

    Dilute stock 1:10 in assay buffer

    (10 uL NPY standard stock + 90 uL assay buffer → **Std A**)

    Dilute Std A 1:100 in assay buffer

    (10 uL Std A + 990 uL assay buffer → **Std B**)

Standard Serial Dilutions					
Standard Number	Solution (uL)		Assay Buffer (uL)	Final Conc. (pg/100 mL)	(ng/mL)
1	10.24	of Std B +	989.76	1024	10.24
2	500	of Std 1 +	500	512	5.12
3	500	of Std 2 +	500	256	2.56
4	500	of Std 3 +	500	128	1.28
5	500	of Std 4 +	500	64	0.64
6	500	of Std 5 +	500	32	0.32
7	500	of Std 6 +	500	16	0.16
8	500	of Std 7 +	500	8	0.08
9	500	of Std 8 +	500	4	0.04

11) After 1 hr, wash plate 3X with 300 uL wash buffer per well. Shake plate over sink to remove solution after each wash. After final wash, invert plate over a paper towel and tap to remove remaining wash buffer.

12) Load plate. Pipette 100 uL assay buffer into NSB and TB wells. Add 100 uL of standards or samples into designated wells (as set up on the plate map) and incubate for 1 hour.
13) Carefully shake solution from plate over the sink. Invert plate over a paper towel and tap gently to remove remaining solution. (**DO NOT WASH**)
14) Obtain 10uL aliquot of Bio-NPY stock solution (stored at -20^{o}C). Dilute as follows.
    Dilute Stock 1:10 in assay buffer
        (10 uL stock + 90 uL buffer → **Bio-NPY A**)
        [Aliquot remaining Bio-NPY A into 10 uL aliquots and store at -20^{o}C]
    Dilute Bio-NPY A 1:100 in assay buffer
        (10 uL Bio-NPY A + 990 uL buffer → **Bio-NPY B**)
    Dilute Bio-NPY C 1:500 in assay buffer
        (20 uL Bio-NPY B + 9980 uL buffer → **Bio-NPY C**)
15) Add 100 uL Bio-NPY C to all wells **EXCEPT** NSB. Cover plate with plastic cover and incubate 2 hours at room temperature.
16) Wash plate 3X as in step 7 above.
17) Make SA-HRP solution by adding 1 uL SA-HRP to 10 mL assay buffer in a 15 mL conical tube. Vortex. Add 100 uL to each well. Cover plate and incubate for 1 hour.
18) Wash plate 3X as in step 7 above.
19) Add 100 uL TMB to each well, cover plate, and incubate for 5-10 minutes. Look for a strong blue color in the TB wells.
20) Stop the reaction by addition of 100 uL 2N H_2SO_4 to each well. Be sure there are no bubbles in the wells. Read plate in plate reader at 450 nm within 30 minutes.