Smith et al. SUPPLEMENTARY MATERIALS AND METHODS

GnIH Radioimmunoassay

TUBES: Plastic 10 x 75 mm

DILUENT: 0.5% BSA Phosphate buffer pH 7.4
For 1 litre:
5g BSA
3.52g NaH_2P0_4 (anhydrous)
17.13G NA_2HP0_4 (anhydrous)
1.0g sodium azide
1ml 10% triton X-100

1^o ANTISERUM: AS1353: Antibody raised against RFRP-3 in guinea pig.

STOCK: 1:50 dilution in 50µl aliquots, prepared from neat stock. To 100ul neat material add 4.9ml assay buffer and aliquot in 50µl lots.

WORKING ANTISERUM: 1:30,000 initial dilution. Dilute 50µl of 1:50 stock to 30ml with 1:2000 normal guinea pig serum (NGPS).

TRACER: ^{125}I mono iodinated GnIH Pep204/hRFRP-3(Tyr^1). Use 15000 cpm per assay tube. Store tracer at -20^{o}C. See separate sheet for iodination procedure.

STANDARD: GnIH Pep204/hRFRP-3(Tyr^1) as per iodination stock diluted to 10ng/ml and aliquoted into 250ul lots which contain 2500pg GnIH.

Take one 250ul aliquot and add 750ul of assay buffer to give top standard which contains 2500pg/ml or 250pg/100ul.
Take 500ul of top standard and dilute with 500ul of assay buffer to double dilute. Continue until you have prepared standard range of 125, 62.5, 31.25, 15.625, 7.183, 3.91, 1.95, 0.98, 0.49pg/100ul.

NORMAL GUINEA PIG SERUM (NGPS):

Working dilution: 1:1000 dilution. Take 50ul neat serum and add to 50mls of assay buffer.

2^o ANTISERUM: Goat anti Guinea Pig Serum (GaGPS) diluted to 1:150 with assay buffer.
Take 1ml neat aliquot and add to 149mls of assay buffer.
(AS439)

QUALITY CONTROLS:

QCs have to be prepared on the day 1 of assay.

1. QC1: Add 4ul of top standard to 1ml of hypox serum x 3 to give ~10pg/ml.
2. QC2: Add 8ul of top standard to 1ml of hypox serum x 3 to give ~20pg/ml
3. QC3: Add 16ul of top standard to 1ml of hypox serum x 3 to give 40pg/ml
4. QC4: Add 24ul of top standard to 1ml of hypox serum x 3 to give ~60pg/ml

All QCs must be extracted along with 3x1ml hypox serum to be used as blank control for extraction procedure

SPIKE: Take 10ul spike aliquot which contains 100pg of GnIH and add to 990ul of assay buffer to give 100pg/ml.
Add 200ul of prepared spike to 1ml hypox serum x 3 to give 20pg/ml.

NOTE: All samples, QCs, Spike and hypox samples have to be extracted prior to RIA.

(See attached extraction procedure).

GnIH RIA Tube Contents:

TUBE		Buffer	STD or Sampler	Tracer	A/S	GaGPS	NGPS	10%PEG
1-2	NSB	300	-	100	-	200	200	100
3-4	Total counts	-	-	100	-	-	-	-
5-6	0 pg/100ul	300	-	100	200	200	-	100
7-8	0.49	200	100	100	200	200	-	100
9-10	0.98	200	100	100	200	200	-	100
11-12	1.95	200	100	100	200	200	-	100
13-14	3.91	200	100	100	200	200	-	100
15-16	7.813	200	100	100	200	200	-	100
17-18	15.625	200	100	100	200	200	-	100
19-20	31.25	200	100	100	200	200	-	100
21-22	62.5	200	100	100	200	200	-	100
23-24	125	200	100	100	200	200	-	100
25-26	250	200	100	100	200	200	-	100
27-28	Sample 1	100	200	100	200	200	-	100
	Hypox	100	200	100	200	200	-	100
	Spike	100	200	100	200	200	-	100
	QCs	100	200	100	200	200	-	100
* 300µl sample may be used, in which case do not add any diluent.								

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Order of Addition:

1. Standards (100µl) and sample (200 µl)
2. Buffer (diluent)
3. First antibody
4. NGPS
5. Incubate 24hrs at 4 ^{o}C
6. Add Tracer
7. Incubate 24hrs at 4 ^{o}C
8. Add Second antibody (GaGPS).
9. Incubate 24hr at 4 ^{o}C
10. Add 100ul 10% Peg, incubate 3hrs at 4 ^{o}C.
11. Spin all tubes except TC tubes at 3000rpm for 30mins.
12. Add 100ul of 5% potato starch, spin further 10mins at 3000rpm.
13. Aspirate supernatant immediately keeping centrifuge buckets on ice.
    Count assay for 2min/tube on gamma counter.

EXTRACTION PROCEDURE FOR PORTAL SAMPLES

* Obtain samples – arrange and label extraction tubes, note numbers and volumes on assay sheets.

* Pipette 1.0ml of sample into numbered tubes – note all variations of volume (0.7ml min volume if possible) so that dilution factor can be applied.

* Pipette out 6 x 1ml aliquots of Hypox (these are for controls and recovery as no GnIH should be present in Hypox plasma).

* Into 3 of the Hypox tubes (e.g. last 3) add 200µl of 100pg/ml GnIH.

* Pipette out 1ml (x 3) of Quality Controls QC1, QC2, QC3 and QC4.
  - these should give readings of 10,20,40 and 60pg/ml of GnIH, respectively.

* Add 4ml Acidified Methanol to all tubes. (1 litre Methanol : 0.6ml Acetic Acid).

* Cap and Vortrex all tubes thoroughly.

* Centrifuge at 3,000cpm, 4^{o}C, for 30min.

* While centrifuge is going, number glass tubes (13 x 100mm) in relation to previous extraction tubes.

* Decant supernatant into corresponding tubes and discard pellet.

* Dry down in Rotary Evaporator (will take overnight to complete).

* Check that all tubes are dry then reconstitute with 1ml buffer. Let tubes stand for 10min, then vortex to dissolve pellet properly.

* Assay this as sample either immediately or store at -20^{o}C (cap all glass tubes).

GnIH IODINATION PROCEDURE:

Preparation

Label scint vials for linear step gradient elution solutions and falcon tubes in same way for fraction collection.

0,5,10,15,20,25,30,35,40,45,50,55,60,65,70,75,80

A. Elution and Reaction Soultions

1. 1% Formic Acid (FA) 250ml

Prepare in fume hood fresh for each iodination:
Add 2.5mls absolute FA to 20ml dH_2O in glass measuring cylinder
Make up to 250ml with dH_2O then transfer to stock bottle

2. 80% Methanol (MeOH) 1% FA 200ml

Prepare in fume hood fresh for each iodination:
Add 2ml absolute FA to 160ml methanol
Make up to 200ml with dH_2O

3. 1% FA 0.1%Bovine Serum Albumin (BSA) 0.1% polypep 10ml

Can be kept at 4ºC for subsequent Iodinations
0.01g BSA and 0.01g polypep in 10ml 1% FA

4. 40% MeOH / 1% FA 0.1% BSA 0.1% Polypep 50ml

Add 0.05g BSA to 25ml 80%MeOH / 1% FA and 25ml dH_2O
Can be kept at 4ºC for subsequent iodinations
Used for storage of Iodinated peptide

5. 0.5mg/ml Chloramine-T 5ml

Prepare fresh immediately before each Iodination:
Make 0.5mg/ml – add 0.25ml of 10mg/ml to 4.75ml dH_2O

6. Linear Step Gradient Elution Solution

Final Dilution MeOH	**80%MeOH**	**1% FA**
0%	-	Soln 1
5% MeOH / 1% FA	0.75 ml	11.25ml
10 "	1.5ml	10.5ml
15 "	2.25ml	9.75ml
20 "	3.0ml	9.0ml
25 "	3.75ml	8.25ml
30 "	4.5ml	7.5ml
35 "	5.25ml	6.75ml
40 "	6.0ml	6.0ml
45 "	6.75ml	5.25ml
50 "	7.5ml	4.5ml
55 "	8.25ml	3.75ml
60 "	9.0ml	3.0ml
65 "	9.75ml	2.25ml
70 "	10.5ml	1.5ml
75 "	11.25ml	0.75ml
80 "	Soln 2	-

B. Activating the Sep-pak

C_{18}–Sep-Pak is activated before commencing each iodination for the use in purification of the iodinated antigen. The cartridge is activated by cycling through the 2 stock elution buffers using a 2 X 3ml syringes (for 80% MeOH / 1% FA and 1% FA) very slowly push each solution through the Sep-Pak as follows:

1. 5ml 80% MeOH
2. 5ml 1% FA
3. 1ml 1% FA, 0.1% polypep (solution 3 above)
4. 5ml 1% FA
5. 2 x 5ml 80%MeOH
6. 2 x 5ml 1% FA

C. Peptide

GnIH-3 hRFRP-3 6.45µg/10µl
Add 10µl 0.5M NaH_2PO_4 (6g in 100ml dH_2O)
Spin 2000rpm 4ºC 5mins

Iodination Reaction

Add 10µl (1mCi) I^{125} on side of tube
Add 10µl Chloramine-T (o.5mg/ml) to other side of tube
Gently tap for 30 seconds
Stop reaction with 1ml FA, 0.1% BSA, 0.1% Polypep (Solution 3)

Purification

The iodinated antigen, I^{125}- hGnIH is immediately crudely purified by reverse chromatography over the activated C_{18}-Sep-Pak using successive 5ml washes of the elution gradient solutions, from 0% MeOH / 1% FA up to 80% MeOH / 1% FA. Each solution is slowly dripped through the column using a 3ml syringe.

Each wash is collected in 15ml falcon tubes. 10µl is collected from each fraction and counted in the gamma counter. Fractions showing peak activity (Usually around 35%, 40%, and 45%) are made up to 10ml with 40% MeOH, 0.1% BSA, 0.1% Polypep (Solution 4) and stored at -20ºC.