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Appendix

RIA protocol for steroid hormones

Day 1

1. Label: glass assay tubes for extraction: samples x2, QC's x2, recoveries x3 (R1, R2 and R3).

   polypropylene assay tubes: similar to glass tubes above plus total recovery counts (TR1, TR2 and TR3), Total counts (TC)x2, std 10x2, Box2, NSBx2)
2. Pipette: add required amount of following samples in glass tubes: samples (100 µl for progesterone and 50 µl for testosterone assay), QCs and charcoal stripped horse serum with around 10,000 cpm/100 µl tracer for recoveries.
3. Extract: add 1.2 ml diethyl ether into every glass tube

   Vortex on sample shaker for 10 min

   Freeze aqueous phase using dry ice

   Decant the unfrozen solvent into corresponding polypropylene tubes

   Evaporate solvent in vacuum oven

   Make a final volume of 100 µl with gel buffer
4. Recovery: add 100 µl of charcoal stripped serum and tracer to Rs and TRs.
5. Standard curve: The standard was serially diluted in halves in gel buffer and 100 µl in each std tube.
6. Bo and NSB: add 100 µl and 200 µl of gel buffer into Bo and NSB, respectively.
7. Add 100 µl of antiserum to the assay tubes except NSB and TC and 100 µl of tracer to all the assay tubes. Vortex
8. Incubate overnight at 4 °C

Day 2

1. Add 100 µl of 10% charcoal stripped serum except TCs.
2. Add 1.5 ml of 22% PEG to the assay tubes except TCs.
3. Centrifuge for 30 min at 4 °C
4. Centrifuge at 3500 rpm for 30 min. aspirate the supernatant.
5. Add 2 ml of scintillation fluid to all assay tubes and recovery tubes. Vortex and incubate for 12-18 hrs.

Day 3

Count for 3 min x 2 / sample

Protocol for Harris's Haematoxylin and Eosin (H & E) staining

Place slides in following for given interval of time

1. Xylol I	5 min.
2. Xylol II	5 min.
3. Absolute alcohol I	3 min.
4. Absolute alcohol II	3 min.
5. 80% alcohol	2 min.
6. 50% alcohol	2 min
7. Distilled water	1 min.
8. Harris's Haematoxylin	15 min.
9. Rinse in tap water	5 min.
10. Acid/alcohol rinse	2 dips
11. Running tap water	30 min.
12. 70% alcohol	2 min.
13. 90% alcohol	2 min.
14. Absolute alcohol III	2 min.
15. Eosin	3 min.
16. Absolute alcohol IV	2 min.
17. Absolute alcohol V	2 min.
18. Xylol III	2 min.
19. Xylol IV	3 min.

20. Mount section in DPX.

Slide Coating

1. Wash slides in detergent for 30 min.
2. Wash slides in running tap water for 30 min.
3. Wash slides in distilled water 2x5 min.
4. Wash glass slides in absolute alcohol for 10 min.
5. Air dry for 10 min.
6. Immerse slides in a freshly prepared 2% solution of 3-aminopropyltriethoxysilane in dry acetone for 10 min.
7. Wash briefly in distilled water
8. Leave slides overnight in dry oven at 37 ^{o}C

Reagents used for indirect ELISA

Binding (Coating) Buffer pH 9.6

Na_2CO_3 (alkaline)	1.59 g
$NaHCO_3$ (acid)	2.93 g
NaN_3 (sodium azide)	2ml of 10%solution)
Distilled water to	1 000 ml

Adjust pH by adding Na_2CO_3 or $NaHCO_3$ (in solution) and store at 4^oC

Wash Buffer

NaCl	90 g
1 M Tris (pH 8.0)	100 ml
Distilled water to	10 L

Blocking solution

5% Skim milk powder in HSE buffer

1% BSA in PBS

High Salt ELISA Buffer (HSE buffer) (pH 8.0)

20mM Tris (Tris Base)	2.42g
0.5 M NaCl	29.22g

0.02% Sodium Azide	2 ml of 10% stock
0.1M $ZnCl_2$	600 µl
1 M $MgCl_2$	600 µl
0.1% BSA	1g
0.5% Tween 20	5ml
Distilled water to	1,000 ml

ELISA Buffer (pH 7.5)

20mM Tris (Tris Base)	2.42g
NaCl	8.76g
0.02% Sodium Azide	2 ml of 10% stock
0.5% BSA	5g
0.1% Tween 20	1ml
Distilled water to	1,000 ml

Enzyme Substrate Buffer (for use in NPP)

Diethanolamine (triethanolamine)	97 ml (133mls)
Distilled Water	800 ml (700mls)
0.02% NaN_3	2 ml of 10% solution
$MgCl_2.6H_2O$	101 mg

Adjust the pH to 9.8 and make up to 1 L with dH_2O. Store at 4°C

(NPP)p-Nitrophenylphosphate disodium salt hexahydrate($C_6H_4NNa_2O_6P*6H_2O$)

1 mg NPP to 1 ml of Enzyme Substrate Buffer.

Reagents used for RIA (steroid hormones)

Gel Buffer

10x PBS	100 ml
gelatine	10 g
NaN_3 (sodium azide)	2 ml of 10% stock
Distilled water to	1000 ml

22% PEG

Polyethylene Glycol (PEG) 6000	550 g
NaCl	22.5 g
Add distilled water to make a final volume of	2 500 ml

Scintillation Fluid

PPO-2,5-Diphenyloxazole	3.0 g
POPOP	0.3 g

(Di-methyl POPOP-1,4-bis[2-(4-methyl-5-Phenyloxazolyl)]-benzene)

Methanol	16 ml
Triton-X 100	50 ml
Toluene	934 ml

Reagents used for SDS-PAGE and Western Blotting

4X Lower Gel Stock Solution (1.5M Tris/HCl + 0.4% SDS)

Tris/HCl	181.7 g
SDS	4.0g

Bring to 900ml with water, adjust pH to 8.8 and make final volume of 1000 ml with distilled water. Filter through 0.45um filter and store refrigerated.

4X Upper Gel Stock Solution (0.5M tris/HCl + 0.4% SDS)

Tris/HCl	60.6g
SDS	4.0 g

Bring to 900ml with water, adjust pH to 6.8 and make final volume to 1000ml with distilled water. Filter through 0.45um filter and store refrigerated.

Acrylamide Stock Solution (30%+0.8% Bis)

Acrylamide	90 g
N,N'-methylene bis acrylamide	2.4g

Bring to 300ml with water, filter through 0.45 um filter and store refrigerated in a foil covered bottle.

10% Ammonium Persulphate

Ammonium persulphate	0.1 g
Add 1 ml water	

Separating Gel (for 2 gels) (12.5% acrylamide)

4x Lower gel stock	5ml
Acrylamide stock	8.35ml
Water	6.66ml
Ammonium Persulfate 10%(10mg/100uL)	50uL
TEMED	5uL

Stacking Gel (for 2 gels) (4.06% acrylamide)

4X Upper gel stock	1.4ml
Acrylamide stock	0.75ml
Water	3.45ml
Ammonium Persulfate 10%	30uL
TEMED	5-10 ul

2x Reducing Sample Buffer

4xupper gel stock	3.75ml

Glycerol	l3ml
2-mercapto-ethanol	1.5ml
SDS	0.9g

Bromophenol blue (small amount to color samples)

Bring to 15ml with water

2x Non-Reducing Sample Buffer

4xupper gel stock	5.6ml
Glycerol	l3ml
SDS	0.9g
Nonidet P-40 detergent (NP-40)	225uL

Bromophenol blue (small amount to color samples)

Bring to 15ml with water

10x Running Buffer

Tris	45.5g
Glycine	216g
SDS	15g

Make up to 1.5 L with water and store at room temperature.

Roeder's Stain

Coomassie R-250	125mg
Isopropanol	125ml
Acetic Acid	50 ml

Combine these ingredients and make final volume to 500ml with distilled water.

Destaining Solution

Acetic acid	175 ml
Methanol	125 ml

Make up to 2500 ml with water.

Gel Shrinking Solution

Methylated spirits	30%
Glycerol	1%

Transfer Buffer

Tris-base	7.575 g
Glycine	36.025
Methanol	375 ml

Bring to 2500 ml with water

Ponceau S Stain

Ponceau S	0.2%
Trichloroacetic acid (TCA)	3%
Sulfosalicyclic acid	3%

Make up to 100 ml in distilled water.

Tris-Buffer (Wash Buffer) pH 8.2-8.4

(20mM Tris-base, 0.15 M NaCl, 0.1% Triton X-100, 0.02% Sodium Azide)

Tris-base	24.22 g
NaCl	8.766 g
Triton X-100	4.5 ml
Sodium Azide	9 ml of 10% stock

Adjust pH to 8.2-8.4 using HCl and make a final volume of 4.5 L with distilled water.

Miscellaneous Buffers

10 x Phosphate Buffered Saline (PBS, 0.5M Phosphate, 1.5M NaCl) pH 7.4

Na_2HPO_4 (141.96) ($Na_2HPO_4.12H_2O$ (MW 358.15))	57.49 g (145.04g)
KH_2PO_4 (136.09)	12.93 g
NaCl (58.44)	87.66 g

Adjust pH to 7.4 and make up to 1L with dH_2O. Dilute 1:10 to prepare 0.05M (1x) PBS.

50% Glycerol Solution

Glycerol	50%
0.5M PBS	50%

Mix and store at room temperature

Homogenization buffer

Benzamidine (5mM)	0.783 g
EDTA (5mM)	1.86 g
NaN_3 (0.02%)	2 ml of 10% stock
Distilled water	1000 ml