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APPENDIX

Chemicals and Solutions

1. 0.1 M phosphate buffer, pH 7.4
   - 1.1 DW 800 ml
   - 1.2 Na_2HPO_4 12.25 g
   - 1.3 NaH_2PO_4 3.68 g
   - 1.4 Adjust pH to 7.4 with HCl
   - 1.5 Add DW to 1,000
2. 0.01% DEPC-DW
   - 2.1 DEPC 100 µl
   - 2.2 DW 1,000 ml
3. 75% ethanol
   - 3.1 Ethanol 100% 75 ml
   - 3.2 DW 25 ml
4. 10X PBS (Phosphate buffered saline) pH 7.4: stock solution
   - 4.1 DW 800 ml
   - 4.2 NaCl 80 g
   - 4.3 $Na_2HPO_4.(2H_2O)$ 14.4 g
   - 4.4 KH_2PO_4 2 g
   - 4.5 Adjust pH to 7.4 with HCl
   - 4.6 Add DW to 1,000 ml
5. 1X (Phosphate buffered saline) pH 7.4: working solution
   - 5.1 10X PBS 100 ml
   - 5.2 DW 900 ml
6. 5% Stacking gel
   - 6.1 40% ACRYLAMIND GEL 375 µl
   - 6.2 1.0M Tris-HCl pH 6.8 380 µl
   - 6.3 10% SDS 30 µl
   - 6.4 TEMED 3 µl
   - 6.5 10% SDS 30 µl
   - 6.6 DW 2.145 ml
7. 12% Running gel

7.1 40% ACRYLAMIND GEL	1.5 ml
7.2 1.5M Tris-HCl pH 8.8	1.25 µl
7.3 10% SDS	50 µl
7.4 TEMED	2 µl
7.5 10% SDS	50 µl
7.6 DW	2.812 ml

8. 4% paraformaldehyde in 0.1M phoosphatse buffer, pH 7.4

8.1 0.1 M PB	1000 ml
8.2 Paraformaldehyde	40 g
8.3 NaOH	2-3 pill

9. 0.3% Triton-X in PBS

9.1 Triton-X	30 ml
9.2 PBS	1,000 ml

10. 0.3% H_2O_2 in methanol

10.1 H_2O_2	30 ml
10.2 Methanol	1,000 ml

11. DAB (working solution)

11.1 Tris-HCL	10 ul
11.2 H_2O_2	10 ul
11.3 DAB	1-2 ug

12. 50 mM Tris-HCL (working solution)

12.1 NH_2 $(CH_2OH)_3 \cdot HCL$	6.057 g
12.2 DW	1,000 ml

13. RIPA lysis buffer

13.1 150 m NaCl	4.383 g
13.2 1% NP-40	5 ml
13.3 0.5% Sodium deoxycholate	2.5 g
13.4 0.1%SDS from 10% SDS	5 ml
13.5 50mM Tris-HCL	3.0285 g
13.6 DW	500 ml

* add 1X protease inhibitor cocktail before used

14. 2x sample buffer

14.1 4% SDS 4 g
14.2 10% 2-mercaptoethanol 10 ml
14.3 20% glycerol 20 ml
14.4 0.004% bromophenol blue 0.004 g
14.5 0.125 M Tris-HCL 1.97 g
14.6 DW 100 ml

15. 1X Tank buffer (pH 8.3)
15.1 Tris (FW121.1) 30.28 g
15.2 Glycine 144.13 g
15.3 SDS 10 g
15.4 DW 10 L

16. Towbin transfer buffer (pH 8.2-8.4)
16.1 Tris (FW121.1) 30.3 g
16.2 Glycine 144.1 g
16.3 SDS 10 g
16.4 DW 8 L
16.5 add Methamol after adjust pH 2 L

17. Tris-buffer saline (TBS)
17.1 Tris-HCL 12.11 g
17.2 DW 900 ml
* adjust pH 7.5
17.3 NaCl 9 g
* dilute to 1 L with DW

18. Tris-buffer saline-Tween (TBS-T)
18.1 TBS 1,000 ml
18.2 0.1%Tween-20 0.01 ml

19. Coomassie blue staining solution
19.1 0.025% Coomassie Brilliant Blue R-250 0.5 g
19.2 40% methanol 800 ml
19.3 7% glacial acetic acid 140 ml
19.4 DW to 2 L

20. Coomassie blue destaining solution I

20.1 40% methanol	400 ml
20.2 7% glacial acetic acid	70 ml
20.3 DW	to 1 L

21. Coomassie blue destaining solution II

21.1 5% methanol	500 ml
21.2 7% glacial acetic acid	700 ml
21.3 DW	to 10 L

22. Ponceau S Staining Solution (0.1%(w/v) Ponceau S in 5%(v/v) acetic acid)

22.1 Ponceau S	1g
22.2 acetic acid	50 ml
22.3 DW	to 1L

Protocols

1. Tissue processing

1.1 Fixation: Fixation, usually by a chemical or mixture of chemicals, permanently preserves the tissue structure for subsequent treatments. Specimen should be immersed in fixative immediately after delivery. Fixation is used to terminate cell metabolism, prevent enzymatic degradation of cell and tissue by autolysis and kill pathogenic organism. The tissue is immersed into 40% formaldehyde in 0.1 M PBS.

1.2 Washing: The tissues are washed in PBS to remove the fixative for 5 minutes.

1.3 Dehydration: Dehydration means the removal of water, a process necessary in two major processes: the preparation of tissue for embedding and sectioning. The majority of dehydrating reagents are serial alcohols (70%-100% of ethanol).

1.4 Clearing: The clearing reagents use for this step in staining have a high index of refraction and render the tissue clear or transparent. Clearing reagents must be miscible with both the dehydrating agent and the infiltration medium, which is most frequently paraffin. Clearing reagents are sometimes referred to as dealcoholization agents; their primary purpose is to remove the alcohol use for dehydration and to make the tissue receptive to the infiltration medium. In this study, clearing reagent is xylene.

1.5 Infiltration: After clearing, the specimen is treated with a liquid embedding medium, paraffin, which infiltrates. The embedding medium holds the cells and intercellular structures in their proper relationship while thin sections are cut.

1.6 Embedding: Embedding also refer to as casting or blocking, involves enclosing the tissue in the infiltration medium use for processing and then allowing the medium to solidify that easy to cut or section.

1.7 Sectioning: Embedding tissues are cut into 5 μm thickness by microtome. Successive sections come off the microtome in a ribbon which mounts on slides.

1.8 Staining: Sections are stained with H&E.

1.9 Mounting: The mounting of sections of stained tissue requires a medium (Permount®) to flow between the slide and the cover slip, fill the tissue and the tissue cavity, release entrapped air bubbles.

These are solutions and times for overnight processing after fixation.

70% Alcohol.....30 min
80% Alcohol.....30 min
85% Alcohol.....30 min
90% Alcohol.....30 min
95% Alcohol.....30 min
95% Alcohol.....30 min
100% Alcohol.....30 min
100% Alcohol.....30 min
Xylene.....1 hour
Xylene.....2 hour
Paraffin.....2 hour
Paraffin.....2 hour

2. Immunohistochemistry technique (IHC)

Protocol:

2.1 Deparaffinize sections and hydrate to distilled water.

2.2 Slides were treated successively with 0.3% Triton-X in PBS to cell permeabilization for 1 hour.

2.3 Wash in PBS for 3 minutes.

2.4 Place slides in 0.3% H_2O_2 in methanol to inhibit intrinsic peroxidase activity for 10 minutes.

2.5 Wash in PBS for 3 minutes.

2.6 Incubate slides with 3-5% fetal bovine serum to prevent nonspecific antibody binding for 30 minutes.

2.7 Wash in PBS for 3 minutes.

2.8 The serial sections were incubated for over night at room temperature with

NRAMP2 rabbit polyclonal IgG, 200 μg/ml (Santa Cruz Biotechmology, CA) at 1:200 dilution, in PBS.

2.9 Wash in three changes of PBS for 5minutes.

2.10 Incubate with biotinylated anti-rabbit IgG (Vector Laboratories; Burlingame, CA) at 1:200 dilutions for 1 hour.

2.11 Wash in three changes of PBS for 5minutes.

2.12 The immunoreaction was developed by incubating with horseradish peroxidase-conjugated streptavidin (Pirece, USA) at 1:200 dilutions for 1 hour. Wash in three changes of PBS for 5minutes and incubating with 0.01% diaminobenzidine tetrahydrochloride (DAB) containing 0.02% H_2O_2 in 50 mM Tris HCl, pH 7.5 for 5-15 minutes.

2.13 Stop reaction by distilled water for 3 minutes

2.14 Counterstained by using Haematoxylin for 7-10 dips and wash in running tap until a slide see clear (for uncounterstained slide can across this step).

2.15 Dehydrate with serial alcohol 70%-95% for 3 minutes and 100% for 5minutes, 2 changes each.

2.16 Clear with 2 changes of xylene for 5 minutes and mount with mounting media (Permount®).

2.17 The immunoreactive reactions were observed and photographed using an Olympus BX50 microscope (Olympus; Tokyo, Japan).

3. Western blotting technique

Protocol:

3.1 Placental tissues (100 g) were homogenized using a Fastprep TMFP120 for 30 second at 4°C in 1 ml RIPA lysis buffer and protease inhibitor.

3.2 Centrifuged at 12,000 rpm at 4°C for 10 minutes.

3.3 The supernatant was taken, the pellet was discarded.

3.4 The protein concentration was measured by spectrophotometry (UV 1650 PC).

3.5 For calibrating with standards, use the 3mg/ml standard protein (BSA) solution to prepare dilutions of 200, 400, 600, 800 and 1000 µg/ml in the same solvent as used to prepare the sample protein. Prepare a blank consisting of solvent alone.

3.6 Set spectrophotometer to 562 nm. Zero the spectrophotometer with the solvent blank. Measure the absorbance of the standards and unknowns.

3.7 If the absorptivity of the protein is known, unknown sample concentration was calculated.

3.8 Prepared each protein sample and boiled with 2x sample buffer.

3.9 The 70 μg sample proteins were loaded on 12% SDS-PAGE for electrophoresis. Run at 200 V and 60mA in SDS-PAGE for 1 hour until the dye is close the bottom.

3.10 Transferred the proteins to a polyvinylidine fluoride (PVDF) membrane (Sigma) at 80 V and 350 mA in Semi-dry transfer for 1 hour. Before blotting, the gel, membrane and filter paper was soaked in transfer buffer

3.11 Blocking with 5% nonfat dry-milk in PBS for overnight and washed with 3 changes of TBS-T for 10 minutes.

3.12 The electrotransferred membrane was incubated with Primary antibody (Mouse anti DMT-1 antibody at 1:200 dilution in PBS for overnight, Mouse anti Actin antibody at 1:500 dilution in PBS for 3 hour)

3.13 Washed with 3 changes of TBS-T for 10 minutes.

3.14 The membrane was incubated with HRP-conjugated secondary antibody 1:2000 dilution in PBS for 1 hour.

3.15 Washed with 3 changes of TBS-T for 15 minutes.

3.16 The labeling reaction was visualized by using an enhanced chemiluminescent substrate (Pierce; Rockford, IL) for 5 minute.

3.17 The membranes were exposed on X-ray film (Eastman Kodak company) for 1 minutes. The autoradiographic blots were quantified by densitometry (si-on image software).

4. Coomascie Blue Staining Method

Protocol:

4.1 Staining gel in staining solution for 4 hours to over night in with gentle agitation

4.2 Destain gel in Destaining solution I. Shahe slowly 30 minutes. This removes the bulk of the exess stain

4.3 Remove Destaining solution I and replace with Destaining solution II. Replenish the solution several times until background of the gel is fully destained.

Store the destained gel in Destaining solution II, add 1% glycerol to last destain before drying the gel.

5. Ponceau S Stain for Western blots

Protocol:

5.1 Incubate the membrane for up to an hour in staining solution with gentle agitation.

5.2 Rinse the membrane in distilled water until the background is clean. The stain can be completely removed from the protein bands by continued washing.

5.3 Stain solution can be re-used up to 10 times.

6. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

6.1 RNA extraction

6.1.1 Placental tissues 50 mg are homogenized using a Fastprap^{TM} FP 120 for 30 sec. at 4°C in 1 ml Trizol reagent.

6.1.2 Add 100 µl chloroform, chilled on ice for 5 min.

6.1.3 Centrifuged at 13,000 rpm at 4°C for 15 min.

6.1.4 The supernatant (RNA) is taken into the new tube.

6.1.5 Added an equal volume of ether and then vertex and centrifuged at 13,000 rpm at 4°C for 5 min.

6.1.6 Discarded the ether by pipette.

6.2 RNA precipitation

6.2.1 Added an equal volume of 2-propanol with the former aqueous solution, vertex and chilled on ice 15 min.

6.2.2 Centrifuged at 13,000 rpm at 4°C for 10 min., then discard 2-propanol.

6.2.3 Washed pallet by cold 75% ethanol 1 ml vertex and centrifuge at 13,000 rpm at 4°C for 7 min.

6.2.4 Discard cold 75% ethanol and keep at room temperature for 30 min.

6.2.5 Dissolve by DEPC-DW 100 µl.

6.3 RNA concentration measurement

6.3.1 Total RNA concentration is measured by spectrophotometer (DU730).

6.3.2 Measured the absorbance of distilled water (DW) which use as the solvent blank.

6.3.3 Dilute each total RNA sample 15 µl in DW 1,485 µl (1:100).

4.3.4 Measure the absorbance at OD 260/280 nm, concentration iscalculated.

6.4 cDNA synthesis

6.4.1 Two micrograms RNA are taken for cDNA synthesis using reverse transcriptase enzymes and oligoprimers and followed the cDNA synthesis kit (RT-PCR kit, Invitrogen).

6.4.2 The cDNA products are stored at -80°C until use.

6.5 PCR amplification

PCR was carried out in a 25 µl reaction mixture containing:

DEPC-DW	19.05	µl
10X PCR running buffer	2.5	µl
50 mM $MgCl_2$	0.75	µl
10 mM dNTP	0.5	µl
Taq polymerase	0.15	µl
Forward Primer	0.5	µl
Reverse Primer	0.5	µl
cDNA products	1	µl

6.6 The PCR amplification is set up to the optimal condition

Initial denerturation	1 cycle	94°C	2 min.
Amplification	40 cycles		
1. Denerturation		94°C	30 sec.
2. Annealing	57°C for β-Actin		1 min.
	65°C for DMT1		
3. Extension	72°C		2 min.
Final extension	1 cycle	72°C	5 min.

6.7 The 10 µl PCR products are loaded on 1% agarose gel to electrophoresis and run at 100 V for 50 min.

6.8 Agarose gel is then stained with ethidium bromide and visualized under UV light.

6.9 The PCR bands are quantified by the densitometer Scion image software analysis.

Gene sequences

1. Homo sapiens solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2), transcript variant 7, mRNA

NCBI Reference Sequence: NM_001174130.1

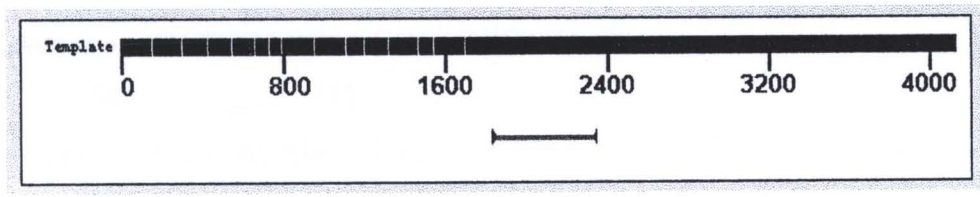
Locus: NM_001174130 4139 bp mRNA linear PRI 17-JUL-2011

Definition: Homo sapiens solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2), transcript variant 7, mRNA.

Accession: NM_001174130

Version: NM_001174130.1 GI:295293177

Summary of primer pairs



product length = 518

Forward primer 1 GAGCCAGTGTGTTTCTATGG 20

Template 1842 1861

Reverse primer 1 CCTAAGCCTGATAGAGCTAG 20

Template 2359 2340

Primer pair 1

	Sequence (5'->3')	Strand on template	Length	Start	Stop	Tm	GC%
Forward primer	GAGCCAGTGTGTTTCTATGG	Plus	20	1842	1861	50.43	50.00%
Reverse primer	CCTAAGCCTGATAGAGCTAG	Minus	20	2359	2340	48.10	50.00%
Internal oligo		Plus					
Product length	518						

2. Homo sapiens actin, beta (ACTB), mRNA

NCBI Reference Sequence: NM_001101.3

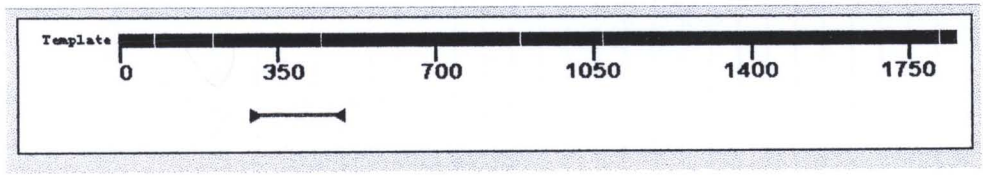
Locus: NM_001101 1852 bp mRNA linear PRI 03-JUL-2011

Definition: Homo sapiens actin, beta (ACTB), mRNA.

Accession: NM_001101

Version: NM_001101.3 GI:168480144

Summary of primer pairs



pro

duct length = 211

Forward primer 1 CATCGAGCACGGCATCGTCA 20

Template 294 313

Reverse primer 1 TAGCACAGCCTGGATAGCAAC 21

Template 504 484

Primer pair 1

	Sequence (5'->3')	Strand on template	Length	Start	Stop	Tm	GC%
Forward primer	CATCGAGCACGGCATCGTCA	Plus	20	294	313	57.54	60.00%
Reverse primer	TAGCACAGCCTGGATAGCAAC	Minus	21	504	484	54.28	52.38%
Internal oligo		Plus					
Product length	211						

Table 15 Data of pregnancy living in non-Cd contaminated area

No.	Maternal weight (kg.)	Maternal hight (m.)	Ferritin ng/mL <16 (2)	Fe (mg/kg) in placenta	Cd (ug/L) in blood	Cd (ug/g crea.) in Urine	Cd (ug/kg) in placenta	Cd (µg/L) in cord blood (8)	Baby weight (kg) (10)
1	78	1.65	18.2	40.35	0.2	1.28	2.5		3.6
2	61	1.56	33	38.47	0.2	1.12	8.5	0.3	2.7
3	84	1.56	20.1	22.74	0.2	1.03	8.5	0.2	3.5
4	67	1.58	14.5	42.4	0.4	1.59	8.5	0.4	3.5
5	59.5	1.60	31.3	51.06	0.8	1.63	12.7	0.2	2.7
6	66.5	1.60	25.9	45.59	0.6	1.67	8.5	0.08	3.4
7	63	1.61	12	23.84	1.4	1.05	8.5		2.8
8	58	1.65	29.3	39.4	0.6	0.9	8.5		2.5
9	68	1.65	18	94.88	0.6	1.78	20.72		
10	52	1.60	8.8	43.61	0.5	0.96	8.5	0.1	2.9
11	55	1.52	13.4	44.64	0.6	0.84	8.5		
12	64	1.58	9.5			0.99			
13	81	1.60	10.9	82.16	0.5	0.65	14.65		
14	64	1.50	18.4	36.53	0.5	0.75	8.8		
15	80	1.65	18.8	58.95	0.5	1.13	8.5	0.3	3.3
16	78	1.62	23.9		0.8	1.26			
17	49	1.55	26.4	79.08	0.5	4.44	8.5		
18	48	1.49	17.9	104.43	0.4	1.19	8.5		
19	61	1.47	6.6	69.06	2	2.02	13.17		
20	67	1.61	20.7	47.79	0.7	1.38	8.5	0.1	
21	72	1.57	19.5	64.14	0.5	0.75	8.5		
22	59	1.60	25.6		0.2	1.84			
23	66	1.62	14	89.92	0.7		2.5		
24			26.1	52.89			8.5		
25			18.8	46.16			8.5		
26			10.6	70.26			8.5		
27			18		2.1	3.78			
28			29.1	96.01	0.5	2.25	8.5		

Table 16 Data of pregnancy living in Cd contaminated area

No.	Maternal weight (kg)	Maternal hight (m)	Ferritin (ng/mL) <16 (2)	Fe (mg/kg) in placenta	Cd (ug/L) in blood	Cd (ug/g crea) in Urine	Cd (ug/kg) in placenta	Cd (µg/L) in cord blood (8)	Baby weight (kg) (10)
1	78	1.65	18.2	40.35	0.2	1.28	2.5		3.6
2	61	1.56	33	38.47	0.2	1.12	8.5	0.3	2.7
3	84	1.56	20.1	22.74	0.2	1.03	8.5	0.2	3.5
4	67	1.58	14.5	42.4	0.4	1.59	8.5	0.4	3.5
5	59.5	1.60	31.3	51.06	0.8	1.63	12.7	0.2	2.7
6	66.5	1.60	25.9	45.59	0.6	1.67	8.5	0.08	3.4
7	63	1.61	12	23.84	1.4	1.05	8.5		2.8
8	58	1.65	29.3	39.4	0.6	0.9	8.5		2.5
9	68	1.65	18	94.88	0.6	1.78	20.72		
10	52	1.60	8.8	43.61	0.5	0.96	8.5	0.1	2.9
11	55	1.52	13.4	44.64	0.6	0.84	8.5		
12	64	1.58	9.5			0.99			
13	81	1.60	10.9	82.16	0.5	0.65	14.65		
14	64	1.50	18.4	36.53	0.5	0.75	8.8		
15	80	1.65	18.8	58.95	0.5	1.13	8.5	0.3	3.3
16	78	1.62	23.9		0.8	1.26			
17	49	1.55	26.4	79.08	0.5	4.44	8.5		
18	48	1.49	17.9	104.43	0.4	1.19	8.5		
19	61	1.47	6.6	69.06	2	2.02	13.17		
20	67	1.61	20.7	47.79	0.7	1.38	8.5	0.1	
21	72	1.57	19.5	64.14	0.5	0.75	8.5		
22	59	1.60	25.6		0.2	1.84			
23	66	1.62	14	89.92	0.7		2.5		
24			26.1	52.89			8.5		
25			18.8	46.16			8.5		
26			10.6	70.26			8.5		
27			18		2.1	3.78			
28			29.1	96.01	0.5	2.25	8.5		

BIOGRAPHY

BIOGRAPHY

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