BGI iTRAQ Quantification Project Report

Bioinformatics Department

Version: 2.1

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Contents

1 MAIN ACHIEVEMENT

1.1 Protein Identification

Table1-1 Protein Identification Achievement Statistics

Group name	Total spectra	Spectra	Unique spectra	Peptide	Unique peptide	Protein
mouse_1	366272	51757	45293	21508	19867	4412
mouse_3	382292	55421	48160	20914	19319	4287
mouse_4	353509	54403	47651	19691	18339	4217

Note: Protein Identification Achievement Statistics. Total Spectra: Total MS/MS Spectras; Spectra: Total Spectras on Identified Proteins; Unique Spectra: Total Unique Spectras on Identified Peptides; Peptide: Identified Peptides; Unique Peptide: Identified Unique Peptide; Protein: Identified Protein.

Outcome Document:

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1.2 Protein Quantification

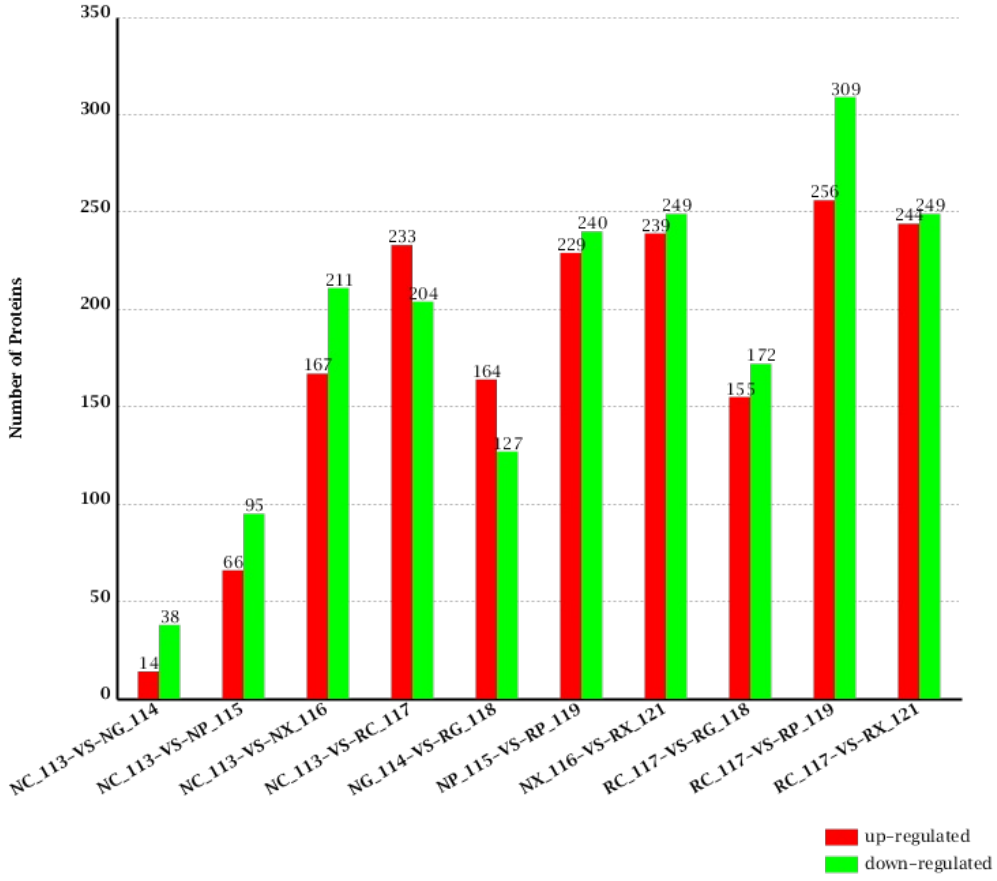
When the fold change is above 1.5 and p-value<0.05, we define this protein is differentially expressed protein.

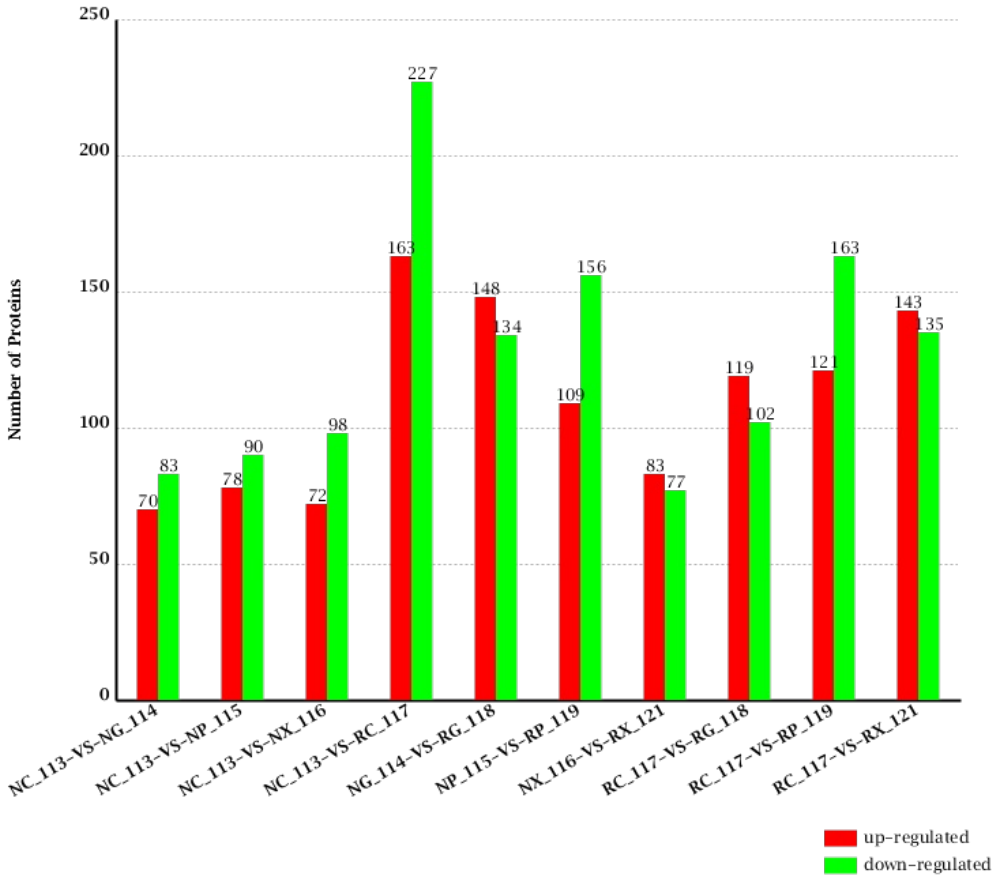
Table1-2 Analysis of Differentially Expressed Protein

Type	UP-regulated	Down-regulated	All-regulated
repeat_1			
NP_115-VS-RP_119	229	240	469
NG_114-VS-RG_118	164	127	291
RC_117-VS-RX_121	244	249	493
RC_117-VS-RG_118	155	172	327

NC_113-VS-NX_116	167	211	378
NC_113-VS-NG_114	14	38	52
NC_113-VS-RC_117	233	204	437
RC_117-VS-RP_119	256	309	565
NC_113-VS-NP_115	66	95	161
NX_116-VS-RX_121	239	249	488
repeat_2			
NP_115-VS-RP_119	109	156	265
NG_114-VS-RG_118	148	134	282
RC_117-VS-RX_121	143	135	278
RC_117-VS-RG_118	119	102	221
NC_113-VS-NX_116	72	98	170
NC_113-VS-NG_114	70	83	153
NC_113-VS-RC_117	163	227	390
RC_117-VS-RP_119	121	163	284
NC_113-VS-NP_115	78	90	168
NX_116-VS-RX_121	83	77	160
repeat_3			
NP_115-VS-RP_119	142	157	299
NG_114-VS-RG_118	116	119	235
RC_117-VS-RX_121	106	49	155
RC_117-VS-RG_118	102	60	162
NC_113-VS-NX_116	98	112	210
NC_113-VS-NG_114	112	151	263
NC_113-VS-RC_117	119	166	285
RC_117-VS-RP_119	82	80	162
NC_113-VS-NP_115	171	212	383
NX_116-VS-RX_121	65	62	127

Note: Differentially expressed protein statistics. Each row is a comparable group.

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156
83
77
119
102
121
163
143
135
NC_113-VS-NG_114
NC_113-VS-NP_115
NC_113-VS-NX_116
NC_113-VS-RC_117
NG_114-VS-RG_118
NP_115-VS-RP_119
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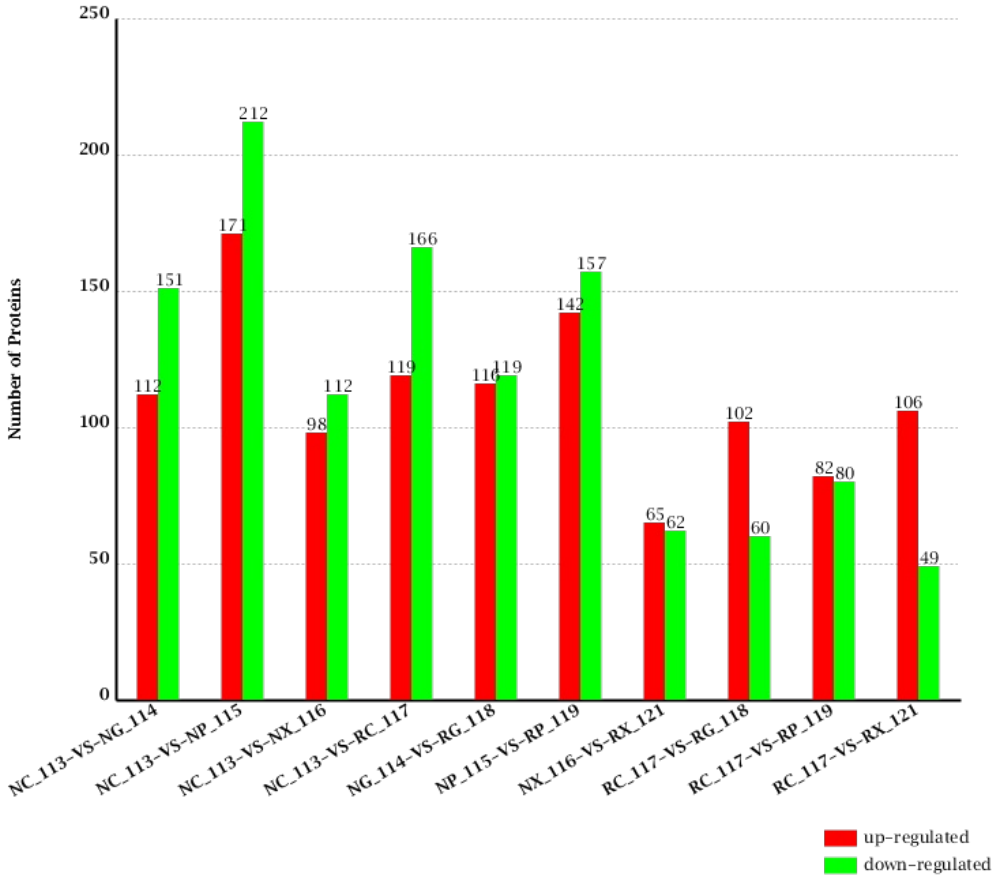
RC_117-VS-RX_121
up-regulated
down-regulated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65
62
102
60
82
80
106
49
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NC_113-VS-NP_115
NC_113-VS-NX_116
NC_113-VS-RC_117
NG_114-VS-RG_118
NP_115-VS-RP_119
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down-regulated

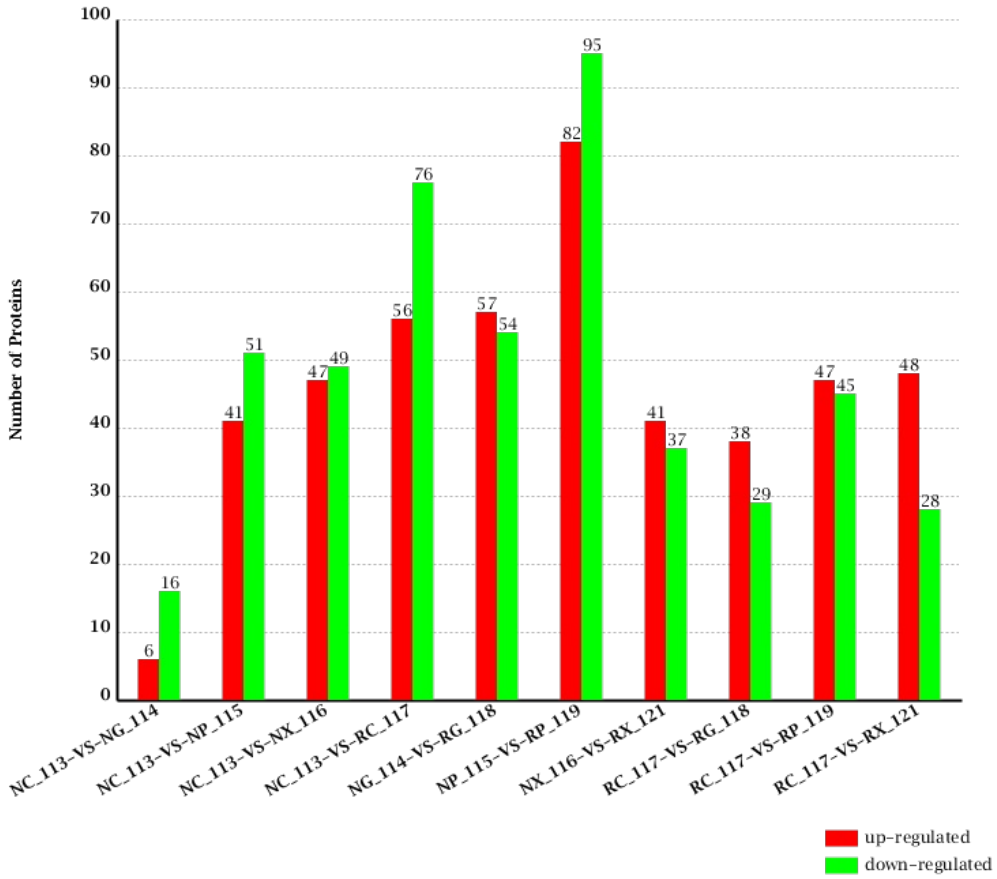

Fig1-1 Differentially Expressed Protein Statistics X-axis: names of comparable group; Y-axis: the number of differentially expressed protein. Red means the number of up-regulated protein, green means the number of down-regulated protein.

Outcome Document:

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2 WORKFLOW DESCRIPTION

The iTRAQ (Isobaric tags for relative and absolute quantitation, iTRAQ) technology has proved to be successful in numerous experimental contexts.This quantitative method can be used for measure eight samples at one experiment and has the characteristics of high precision.So far,it has been more and more widely used in the field of quantitative proteomics.

2.1 Experimental Procedures

2.1.1 Standard Experimental Procedures

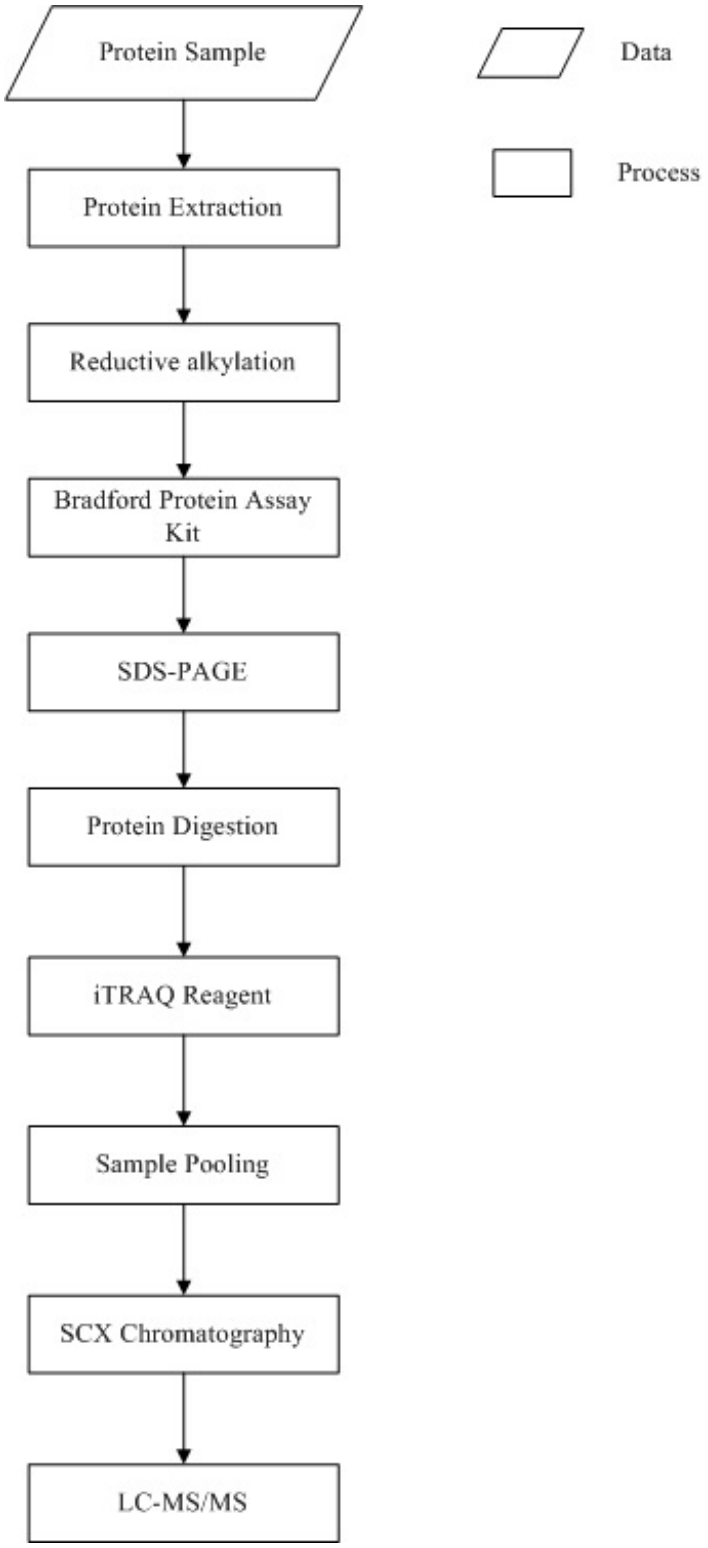

Fig2-1 Experimental Procedures This figure shows the main procedures of the experiment of iTRAQ quantitative proteomics. (1) Extract protein from samples. (2) Reductive alkylation. (3) Measuring the concentration of the protein with Brandford method. (4) SDS-PAGE. (5) Digest proteins in gel and elute peptides. (6) iTRAQ Labeling. (7) Mix peptides with a proportion of 1:1. (8) SCX Chromatography. (9) Liquid chromatography coupled with tandem mass spectrometry(LC-MS/MS).

2.1.2 Protein Extraction

1. Takgh an appropriate amount of tissue and grind them to powder in liquid nitrogen.

2. Add suited volume of Lysis buffer and add PMSF,EDTA to 1mM and 2mM final concentration respectively, after 5 minutes,add DTT to 10mM final concentration.

3. Sonicate the suspension for 15 minutes and then centrifuge 25,000×g for 20minutes.

4. Reduce the disulfide bond of supernatant with 10 mM DTT at 56 for 1 hour.

5. Block the cysteine with 55 mM IAM in dark room for 45minutes.

6. Add five-fold chilled acetone into the supernatant for 2 hours at -20 .

7. Discard the supernatant after centrifugation 25,000×g for 20 minutes and dry the pellet in the air for 5min.

8. Dissolve the pellet in 200μl 0.5M TEAB and sonicate it for 15 minutes.

9. Centrifuge 25,000×g for 20 minutes and the supernatant is quantified in the next step.e a suitable amount of sample;

2.1.3 Protein Concentration Measurement

Measuring the concentration of the protein with Brandford method.

1. Prepare for a standard curve of BSA. 9 tubes contained standard BSA(0.2μg/μl BSA) sequentially(0,2,4,6,8,10,12,16,20μl), pure water was added to each tube aiming to the same volume(20μl). The tube 1 is protein-free(reference),others containing 0.4,0.8,1.2,1.6,2,2.4,3.2,4μg protein respectively .

2. To each tube,add 180μl protein assay reagent, mixed and incubated for 10 minutes at room temperature.

3. The absorbance at 595nm was readed using a microplate reader, tube 1 as reference.

2.1.4 SDS-PAGE

According to the result of concentration, take out 30μg protein from each sample and mix briefly in equivalent loading buffer at 95 in heat block for 5 minutes. Load one sample onto one well and load 10μl marker standard onto this gel, electrophoresis for 2 hours on 120 Volt. The concentration of gel was 12%. When electrophoresis is completed, the gel is dyed by dyeing buffer for 2 hours and destained by destain buffer for 30 minutes 3-5times next.

2.1.5 Protein Digestion

1. Take out 100μg protein for treatment from each sample solution accurately.

2. Digest the protein with Trypsin Gold with the ratio of protein :trypsin =20 :1 at 37 for 4 hours.

3. Add Trypsin Gold with the ratio of protein : trypsin = 20 :1 once more and digest for 8 hours unceasingly.

2.1.6 iTRAQ Labeling

1. After trypsin digestion, the peptides vacuum centrifuged to dryness.

2. Redissolve with 0.5M TEAB ,The iTRAQ labeling of peptide samples were performed using iTRAQ Reagent 8-plex Kit according to the manufacturer's protocol.

3. The peptides labeled with respective isobaric tags, incubated for 2h.

4. The iTRAQ labeled peptides were fractionated using SCX.

2.1.7 SCX Chromatography

For SCX chromatography using the Shimadzu LC-20AB HPLC Pump system, the peptide from digestion is reconstituted with 4 mLbuffer A(25mM NaH2PO4 in 25% ACN, pH2.7)and loaded onto a 4.6×250 mm Ultremex SCX column containing 5-μm particles(Phenomenex). The peptides is eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5-35% buffer B(25 mM NaH2PO4, 1M KCl in 25% ACN, pH2.7)for 11 min,35-80% buffer B for 1 min.The system is then maintained in 80% bufferB for 3 min before equilibrating with buffer A for 10 min prior to the next injection.

Elution is monitored by measuring absorbance at 214 nm, and fractions are collected every 1 min. The eluted peptides are pooled as 20 fractions,desalted by Strata X C18 column(Phenomenex)and vacuum-dried.

2.1.8 LC-ESI-MSMS analysis based on Triple TOF 5600

Each fraction is resuspended in certain volume of buffer A (2% ACN, 0.1%FA)and centrifuged at 20000g for 10min. In each fraction, the final concentration of peptide is about 0.5ug/ul on average. 10ul supernatant is loaded on an Shimadzu LC-20AD nanoHPLC by the autosampler onto a 2cm C18 trap column (inner diameter 200 μm) and the peptides are eluted onto a resolving 10cm analytical C18 column (inner diameter 75 μm) made in-house. The samples are loaded at 15 μL/min for 4 min, then the 44 min gradient is run at 400 nL/min starting from 2 to 35% B (98%ACN, 0.1%FA), followed by 2 min linear gradient to 80%, and maintenance at 80% B for 4 min, and finally return to 2% in 1 min.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX,Concord,ON) fitted with a Nanospray III source (AB SCIEX, Concord,ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI,nebulizer gas of 15 PSI, and an interface heater temperature of 150 °C. The MS was operated with a RP of greater than or equal to 30 000 FWHM for TOF MS scans. For IDA,survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts per second (counts/s) and with a 2+ to 5+ charge-state. Total cycle time was fixed to 3.3s. Q2 transmission window was 100Da for 100%. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode channel detect ion. A sweeping collision energy setting of 35±5 eV adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (18 s), and then the precursor was refreshed off the exclusion list.

2.1.9 Others

iTRAQ experimental protocol：

http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-4370075B.pdf

iTRAQ reagent direction：

http://www.sciex.com/products/standards-and-reagents/itraq-reagents

2.2 Quantitative Proteomics

iTRAQ technology was firstly proposed at the USA MS conference in 2004. It has been wildly used.In 2009, more than 150 articles adopt this technology and it has been increasing year by year.

2.2.1 iTRAQ Reagent Structure

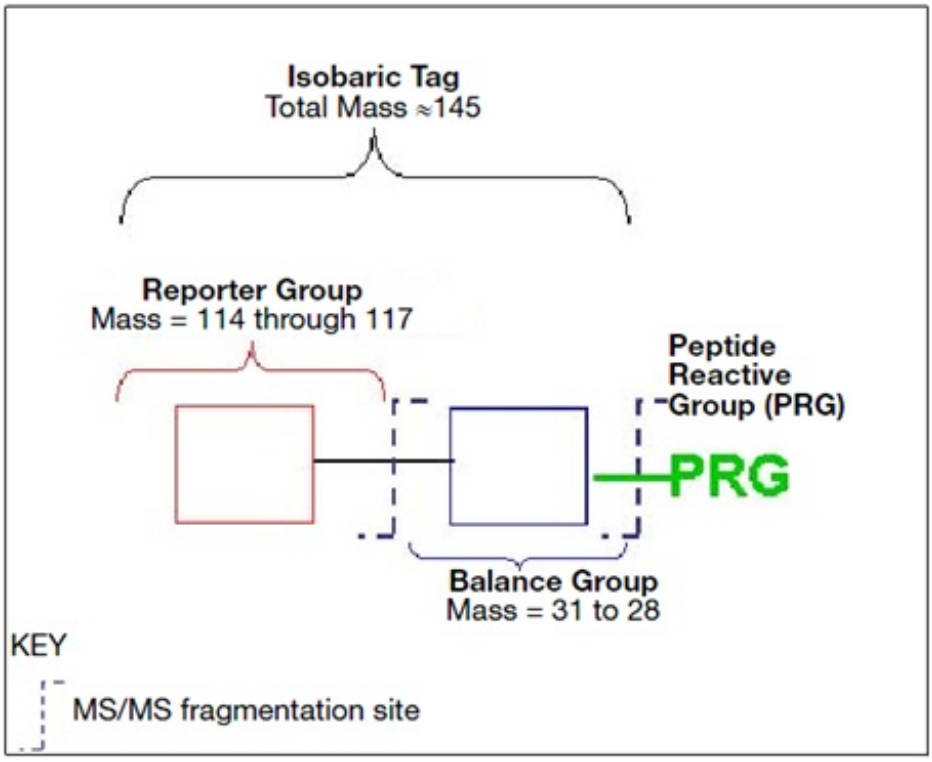

Fig2-2 iTRAQ reagent structure iTRAQ reagent consist of report group，balance group and peptide reactive group. Reactive group can react with N-terminal of peptides and ε-amino group of Lys residue. So it can label any peptide.

2.2.2 The Principle of iTRAQ Quantitative Proteomics

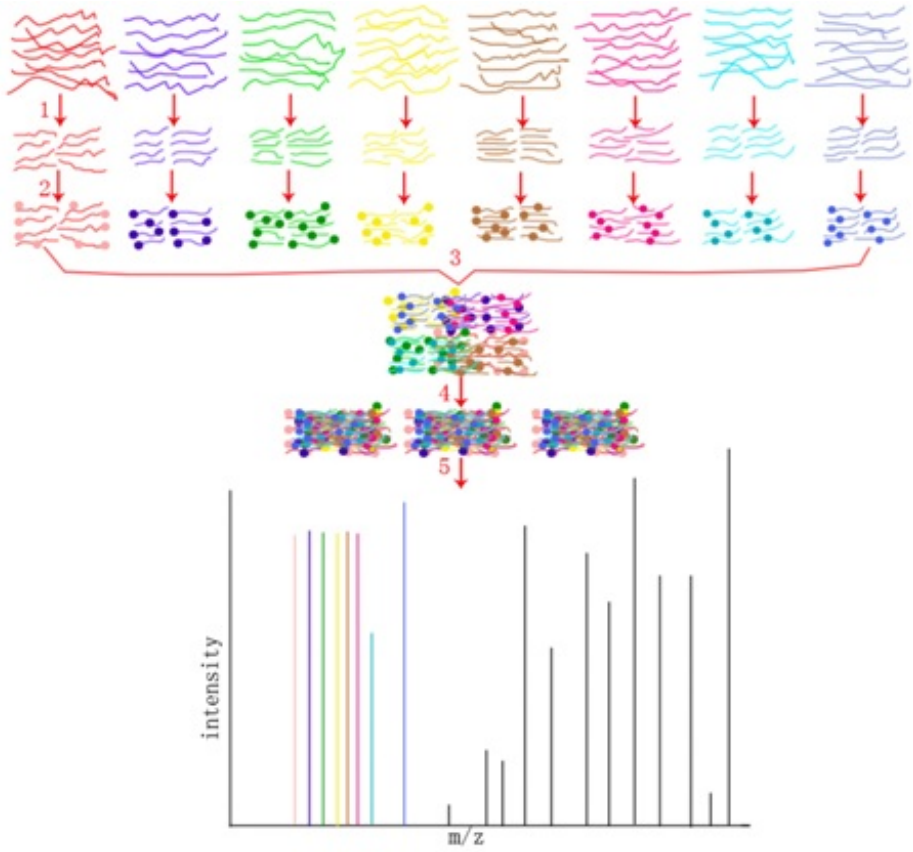

Fig2-3 The principle of iTRAQ quantitative proteomics This picture shows the basic principle of iTRAQ quantitative

proteomics and the main steps of quantitative techniques. iTRAQ quantitative method can compare the relative protein of 8 samples simultaneously at a mass spectrometric experiment. As the figure shows, the process as follow: protein extraction, enzymolysis, iTRAQ lable, mixed, SCX separation and LC-MS/MS. In MS, balance group can show the same M/Z no matter which report ion leble peptide. In MS2, neutral loss happened to balance group, the intensity of report ion can reflect the the relative abundance of the peptides. In the bottom of the figure, there is a MS/MS. X-axis is M/Z, Y-axis is the intensity of ion. The eight colour peaks express the 8 report ions of iTRAQ. The height shows the relative amont of peptides, which can be used to the subsequent quantification. Other black peaks are the MS/MS ion peaks of peptide fragmentations for subsequent identification.

The advantages of iTRAQ: (1)Because iTRAQ reagent can mark any peptide, containing post-translational modifications of peptides, it can improve the reliability and coverage of protein identification. (2)It can improve the reliability of quantifacation, because it can quantify mass peptide of a protein. (3)It's a high-flux research method on discovery of biomarkers. (4)High quantitative accuracy. (5)It can compare the relative protein of 8 samples simultaneously. Base on the method of one same sample, it can compare the quantification of more than 8 samples.

2.3 Bioinformatics Analysis Procedures

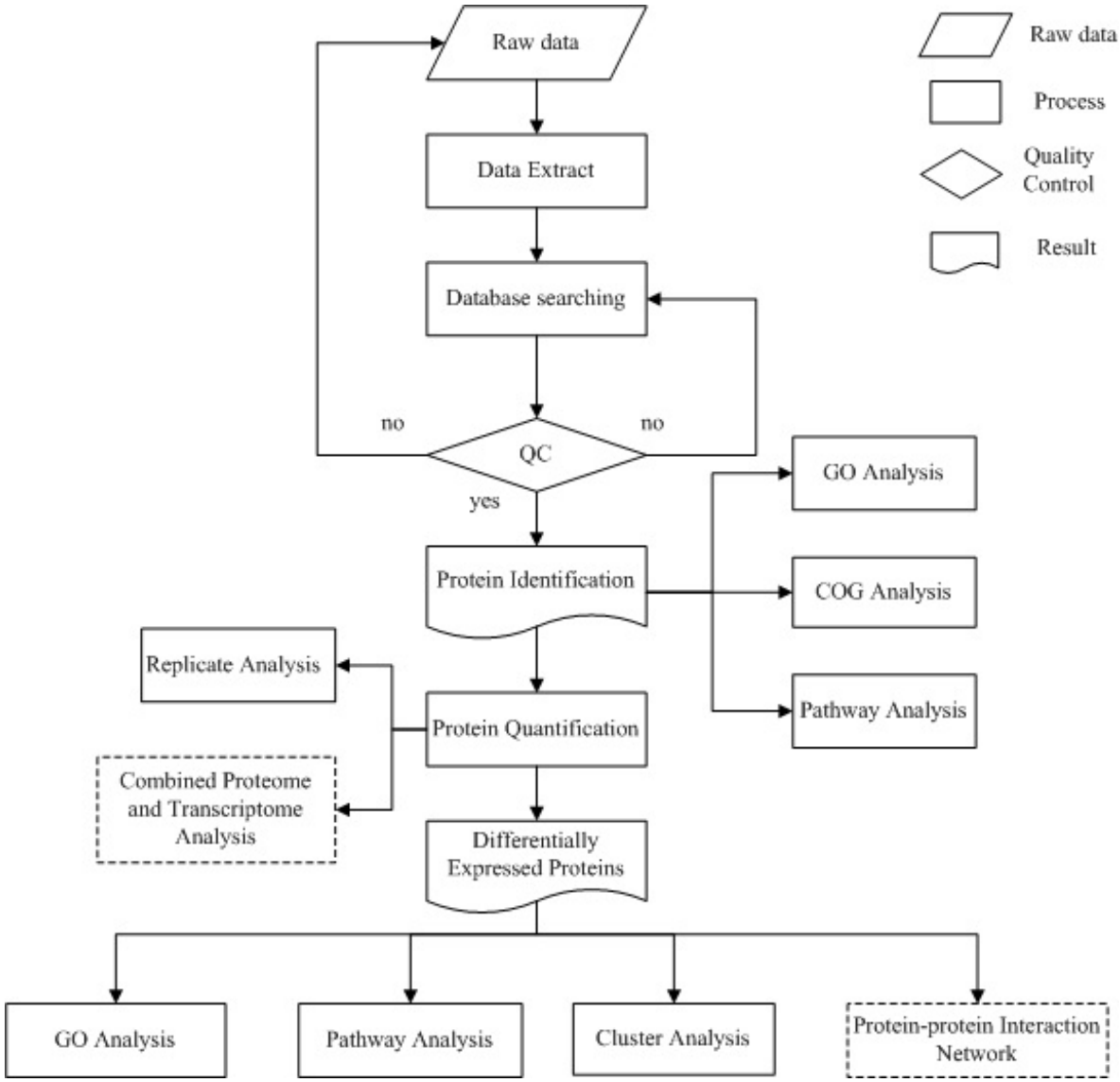

Fig2-4 Bioinformatics Analysis Procedures This figure shows the basic information Procedures.Firstly, identify the peak of the Raw Data , get the peak list. Then, eatablish the database and identify the peptide and protein. At last, compare the relationship of relative amount between the different samples, consequently get some important protein of

interest. We also can combined proteome data with transcriptome data to get the combined proteome and transcriptome analysis.

3 EXPERIMENTAL TESTING

Concentration of protein sample. statistical information is as follows:

Table 3-1 Sample Information

Sample ID	Sample Name	Concentration(μg/μl)	Volume(μl)	Total Protein(μg)
P1305620001	NC1	5.22	110	574.63
P1305620002	NC2	3.59	110	395.37
P1305620003	NC3	8.44	110	928.65
P1305620004	NG1	4.61	200	922.56
P1305620005	NG2	4.03	110	443.40
P1305620006	NG3	3.81	110	418.78
P1305620007	NP1	2.91	110	320.30
P1305620008	NP2	2.33	110	256.29
P1305620009	NP3	2.31	110	253.83
P1305620010	NX1	2.36	110	259.98
P1305620011	NX2	4.19	110	460.63
P1305620012	NX3	4.56	110	501.25
P1305620013	RC1	1.48	110	162.74
P1305620014	RC2	9.25	220	2034.56
P1305620015	RC3	5.58	110	613.52
P1305620016	RG1	5.34	110	587.42
P1305620017	RG2	6.20	110	682.45
P1305620018	RG3	5.13	110	564.28
P1305620019	RP1	5.87	220	1290.56
P1305620020	RP2	4.22	110	464.32
P1305620021	RP3	4.99	110	549.26
P1305620022	RX1	3.95	110	434.78
P1305620023	RX2	4.43	110	487.71

P1305620024	RX3	3.82	110	420.00

4 STANDARD BIOINFORMATICS ANALYSIS

4.1 Raw Data

Raw data must be converted to mgf files for bioinformatics analysis. For an MS/MS Ions Search, each query represents a complete MS/MS spectrum, and is delimited by a pair of statements: BEGIN IONS and END IONS.For example：

BEGINIONS

TITLE=Spectrum1 scans: 2,

PEPMASS=588.84003 11629.05371

CHARGE=2+

RTINSECONDS=0

SCANS=2

115.55481 258.814

116.11040 496.234

136.94980 420.235

173.90498 377.256

180.86188 368.726

190.94720 352.431

……

ENDIONS

The MGF format like this:

TITLE Query title(Applies to a single spectrum)

PEPMASS Peptide mass(optionally followed by intensity)

CHARGE Peptide charge

RTINSECONDS Retention time or range (in seconds)

SCANS Scan number or range

[source]:http://www.matrixscience.com/help/data_file_help.html

4.2 Database for Identification

Protein identification from tandem mass spectra by database searching is widely adopted. The appropriate choice of the sequence database is important for successful application of this method. The general guidelines for database selection like this:

The single species protein databases are good for protein identification.If there hasn't the species protein database, we will choose a larger database about it. In addition, genome database and trancriptome database are good for protein identification.

The common protein databases are:

1. NCBInr: The nr database is compiled by the NCBI (National Center for Biotechnology Information) as a protein database for Blast searches. It contains non-identical sequences from GenBank CDS translations, PDB, Swiss-Prot, PIR,

and PRF.

2. SwissProt: Swiss-Prot is acknowledged to be the best annotated database,but it is non-redundant.

3. UniProt: It provide the scientific community with a comprehensive,high-quality and freely accessible resource of protein sequence and functional information.

4. others.

Database of the project:uniport mouse(After Redundat 79037 sequences)

Database link:http://www.uniprot.org/uniprot/?query=taxonomy%3a10090&format=*&compress=yes

4.3 MS/MS Ion Search

Mascot is a protein identification software which is awarded as the gold standard for bioinformatics by Frost/Sullivan research organization.

The MGF files are searched using Mascot version 2.3.02 in this project against the selected database.

The parameters are as follows:

Table4-1 Mascot Search Parameters

Item	Value
Type of search	MS/MS Ion search
Enzyme	Trypsin
Fragment Mass Tolerance	±0.1Da
Mass Values	Monoisotopic
Variable modifications	Gln->pyro-Glu (N-term Q),Oxidation (M),iTRAQ8plex (Y)
Peptide Mass Tolerance	±0.05Da
Instrument type	Default
Max Missed Cleavages	1
Fixed modifications	Carbamidomethyl (C),iTRAQ8plex (N-term),iTRAQ8plex (K)
Database	uniport mouse(After Redundat 79037 sequences)

4.4 Quality Control

4.4.1 Mass Error Distribution

The AB SCIEX TripleTOF 5600 System is an innovative breakthrough in LC-MS/MS performance that uniquely integrates comprehensive qualitative exploration, rapid profiling, and high-resolution quantitation workflows on a single platform. It combines the highest sensitivity detection, high-resolution with at least 5X better acquisition speed, and stable ~1ppm mass accuracy over days of acquisition.

[source]:http://www.absciex.com/products/mass-spectrometers/tripletof-systems/ab-sciex-tripletof-5600-system

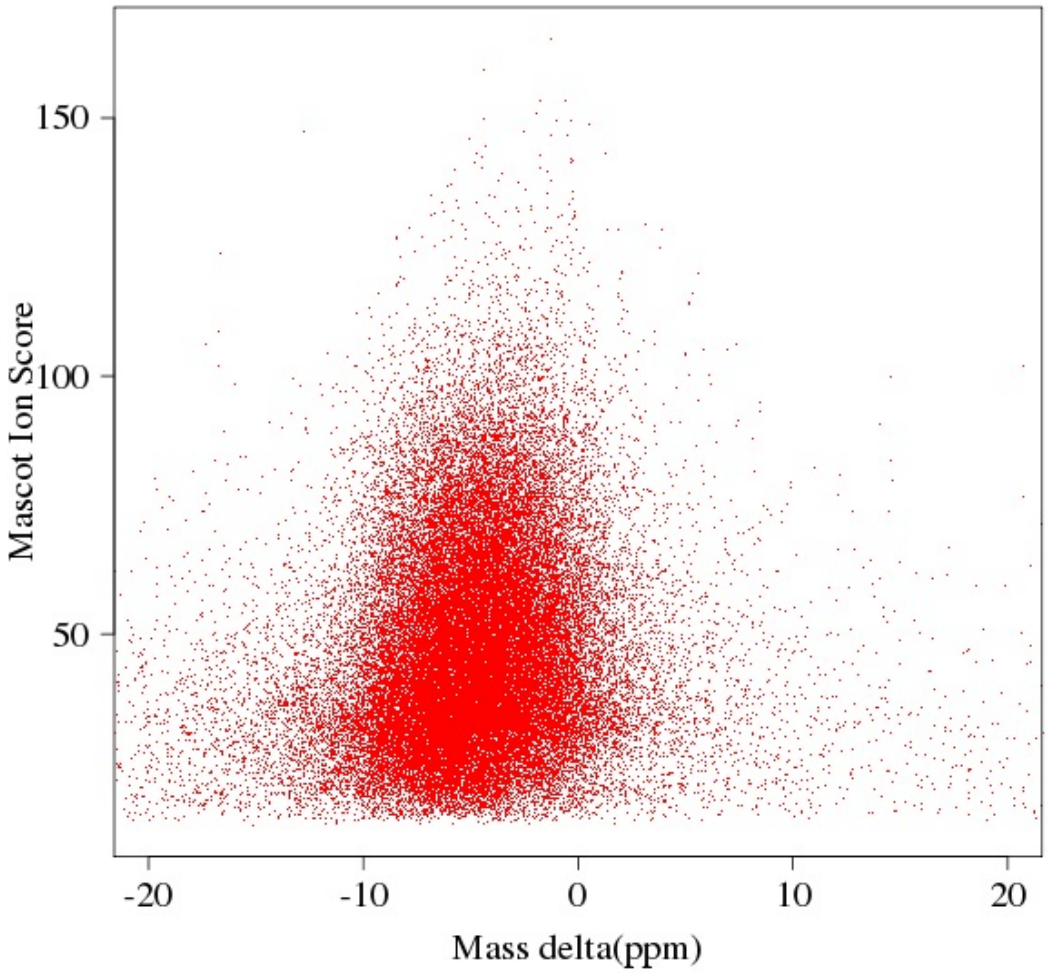

Fig4-1 Mass Error Distributoin

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/mass_delta.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/mass_delta.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/mass_delta.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/mass_delta.png

4.5 Protein Identification

4.5.1 Identification Overview

Table4-2 Identification Overview

Group name	Total spectra	Spectra	Unique spectra	Peptide	Unique peptide	Protein
mouse_1	366272	51757	45293	21508	19867	4412
mouse_3	382292	55421	48160	20914	19319	4287
mouse_4	353509	54403	47651	19691	18339	4217

Note:Protein Identification Achievement Statistics. Group name: category; Total Spectra: Total MS/MS Spectras; Spectra: Total spectras on Identified Proteins; Unique Spectra: Total Unique Spectras on Identified Peptides; Peptide: Identified Peptides; Unique Peptide: Identified Unique Peptide; Protein: Identified Protein.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Identification/all_protein.fa

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Identification/all_protein_annot.xls

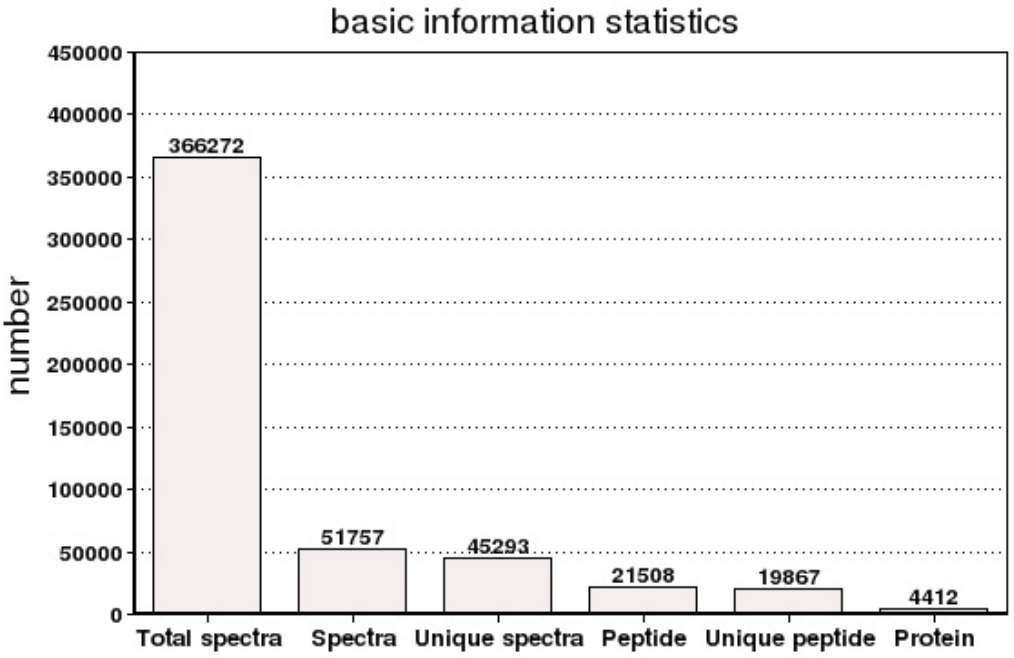

Fig4-2 Identification Overview Note:Protein Identification Achievement Statistics. Group Name: Category; Total Spectra: Total MS/MS Spectras; Spectra: Total Spectras on Identified Proteins; Unique Spectra: Total Unique Spectras on Identified Peptides; Peptide: Identified Peptides; Unique Peptide: Identified Unique Peptide; Protein: Identified Protein.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/basic.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/basic.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/basic.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/basic.png

4.5.2 Protein Molecular Weight Distribution

Statistical Protein base on Molecular Weight

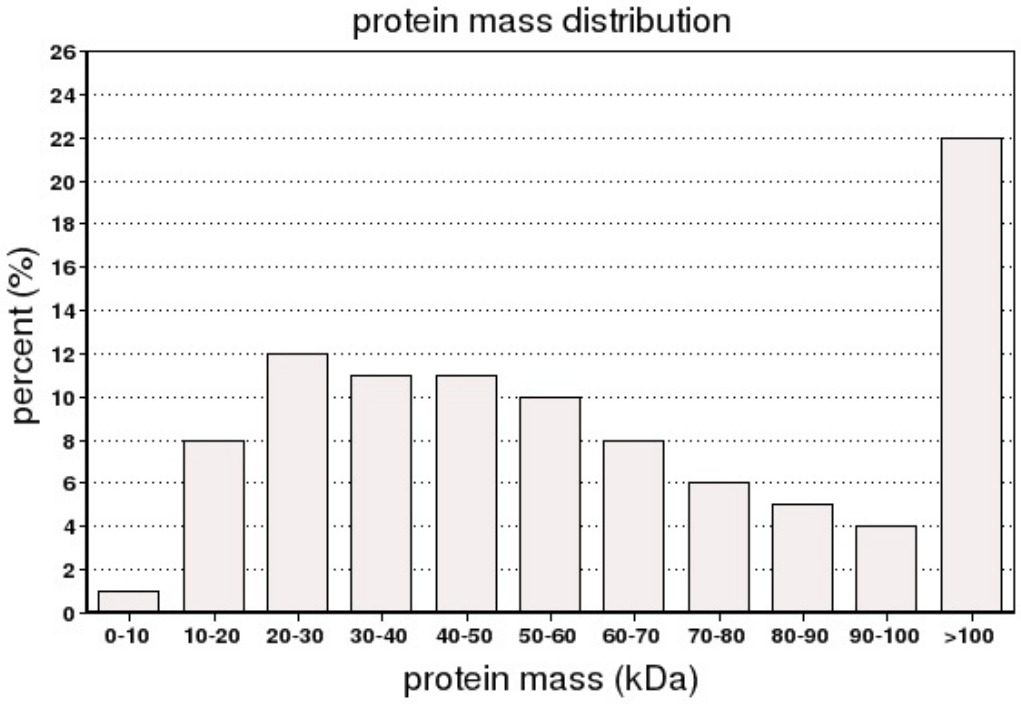

Fig4-3 Protein Molecular Weight Distribution X-axis:Molecular Weight(kDa), Y-axis:Number of Proteins.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/proteinMassStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/proteinMassStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/proteinMassStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/proteinMassStat.png

4.5.3 Peptide Length Distribution

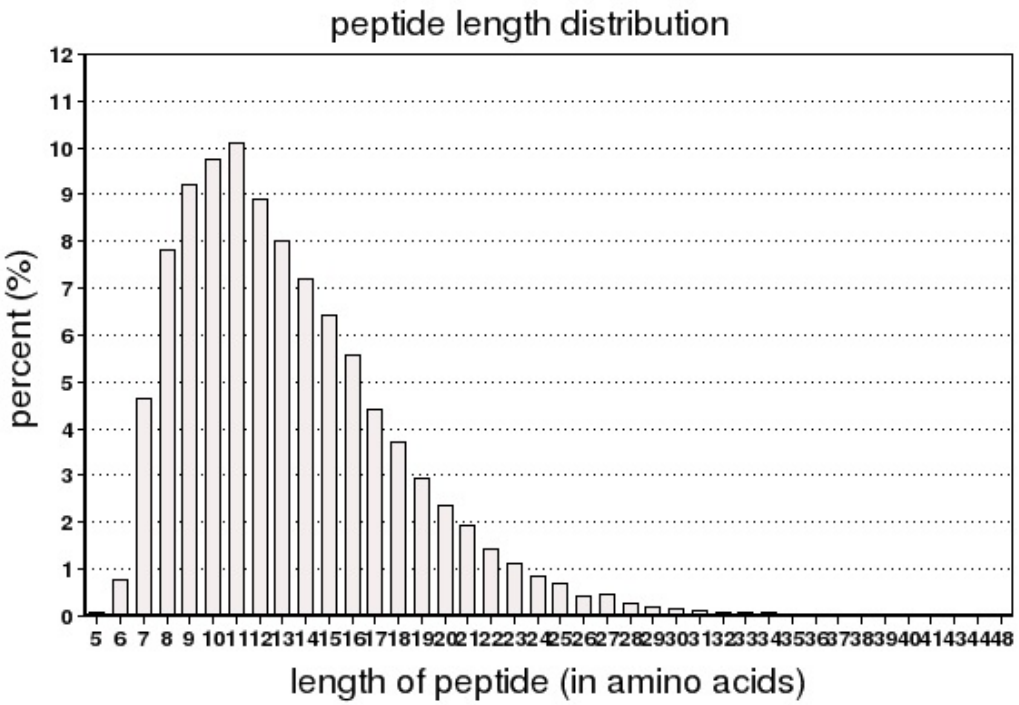

Fig4-4 Peptide Length Distribution This picture shows the percent of different length of peptides. X-axis: the peptide length, Y-axis: the corresponding peptide count.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/peptideLengthStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/peptideLengthStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/peptideLengthStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/peptideLengthStat.png

4.5.4 Sequence Coverage Distribution

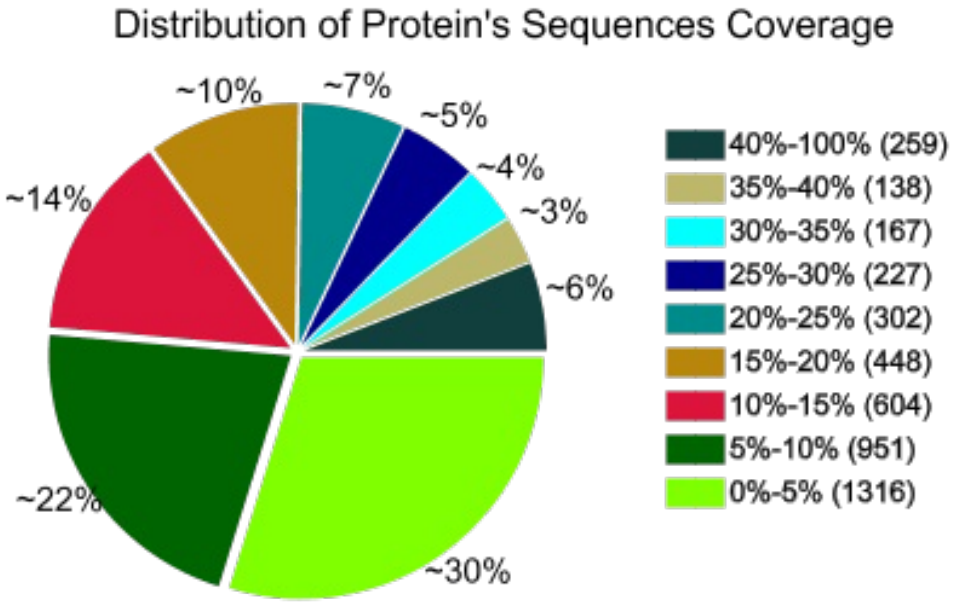

Fig4-5 Sequence Coverage Distribution This picture shows the ratio of different coverage of protein.Different color represents different sequence coverage range. The percentage of the pie chart shows the ratio between protein quantity of different coverage and total protein quantity.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/cover.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/cover.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/cover.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/cover.png

4.5.4 Unique Peptide Distribution

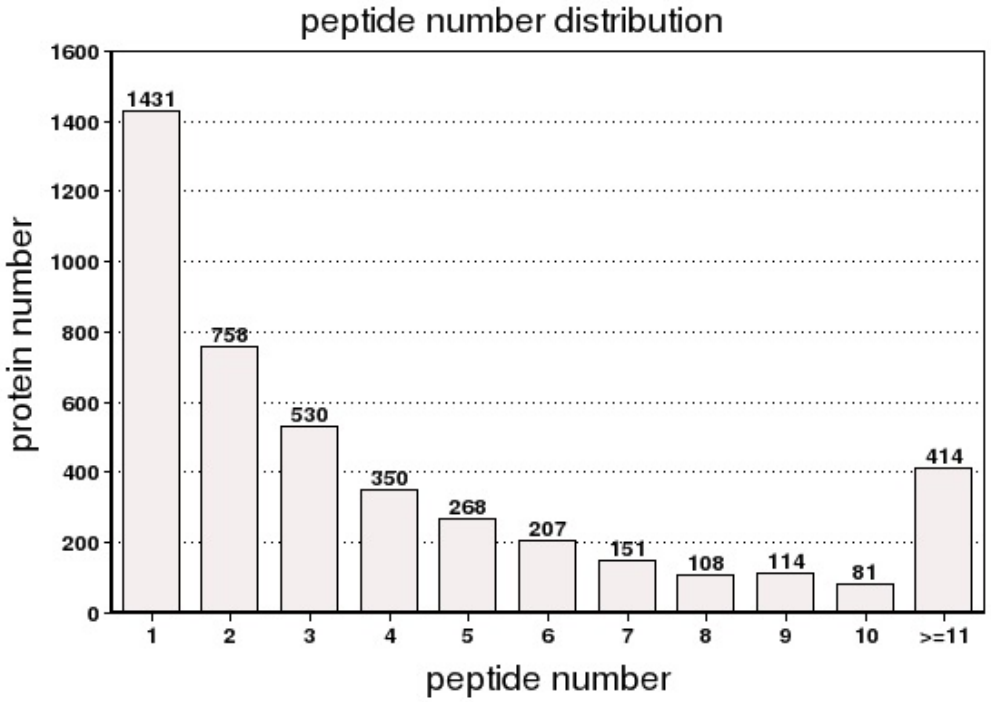

Fig4-6 Unique Peptide Number Distribution It shows the distribution of the peptide quantity which is identified in protein. The X-axis shows the coverage of protein peptide and the Y-axis shows protein quantity. The trend of this picture means that most of the identified protein contain less than 10 peptides and protein quantity become less with the increase of peptide.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/peptideNumberStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/peptideNumberStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/peptideNumberStat.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/peptideNumberStat.png

The results of the identified protein in the files of "sample_detail_annot.xls" and "sample_overall_annot.xls". The file of "sample_detail_annot.xls" contains the informations of peptide and protein. "sample_overall_annot.xls" contains the informations of protein. "sample.fa" regards to the information of protein sequence.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Identification/mouse_1_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Identification/mouse_3_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4.fa

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4_detail_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Identification/mouse_4_overall_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Identification/all_protein.fa

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Identification/all_protein_annot.xls

4.6 Protein Quantification

4.6.1 iTRAQ Labeling Information

Table4-3 iTRAQ Labeling Information

Sample Name	Mark NO
NC	113
NG	114
NP	115
NX	116
RC	117
RG	118
RP	119
RX	121

4.6.2 Protein Quantification Information

Base on the abundance of the protein, when the fold change is above 1.5 and p-value<0.05, we define this protein is differentially expressed protein.

Table4-4 Protein Quantification Information

Type	UP-regulated	Down-regulated	All-regulated
repeat_1			
NP_115-VS-RP_119	229	240	469
NG_114-VS-RG_118	164	127	291
RC_117-VS-RX_121	244	249	493
RC_117-VS-RG_118	155	172	327
NC_113-VS-NX_116	167	211	378
NC_113-VS-NG_114	14	38	52
NC_113-VS-RC_117	233	204	437
RC_117-VS-RP_119	256	309	565
NC_113-VS-NP_115	66	95	161

NX_116-VS-RX_121	239	249	488
repeat_2			
NP_115-VS-RP_119	109	156	265
NG_114-VS-RG_118	148	134	282
RC_117-VS-RX_121	143	135	278
RC_117-VS-RG_118	119	102	221
NC_113-VS-NX_116	72	98	170
NC_113-VS-NG_114	70	83	153
NC_113-VS-RC_117	163	227	390
RC_117-VS-RP_119	121	163	284
NC_113-VS-NP_115	78	90	168
NX_116-VS-RX_121	83	77	160
repeat_3			
NP_115-VS-RP_119	142	157	299
NG_114-VS-RG_118	116	119	235
RC_117-VS-RX_121	106	49	155
RC_117-VS-RG_118	102	60	162
NC_113-VS-NX_116	98	112	210
NC_113-VS-NG_114	112	151	263
NC_113-VS-RC_117	119	166	285
RC_117-VS-RP_119	82	80	162
NC_113-VS-NP_115	171	212	383
NX_116-VS-RX_121	65	62	127

4.6.3 Analysis of Differentially Expressed Protein

Comparative analysis two samples , the number of differentially expressed protein was showed as follows.

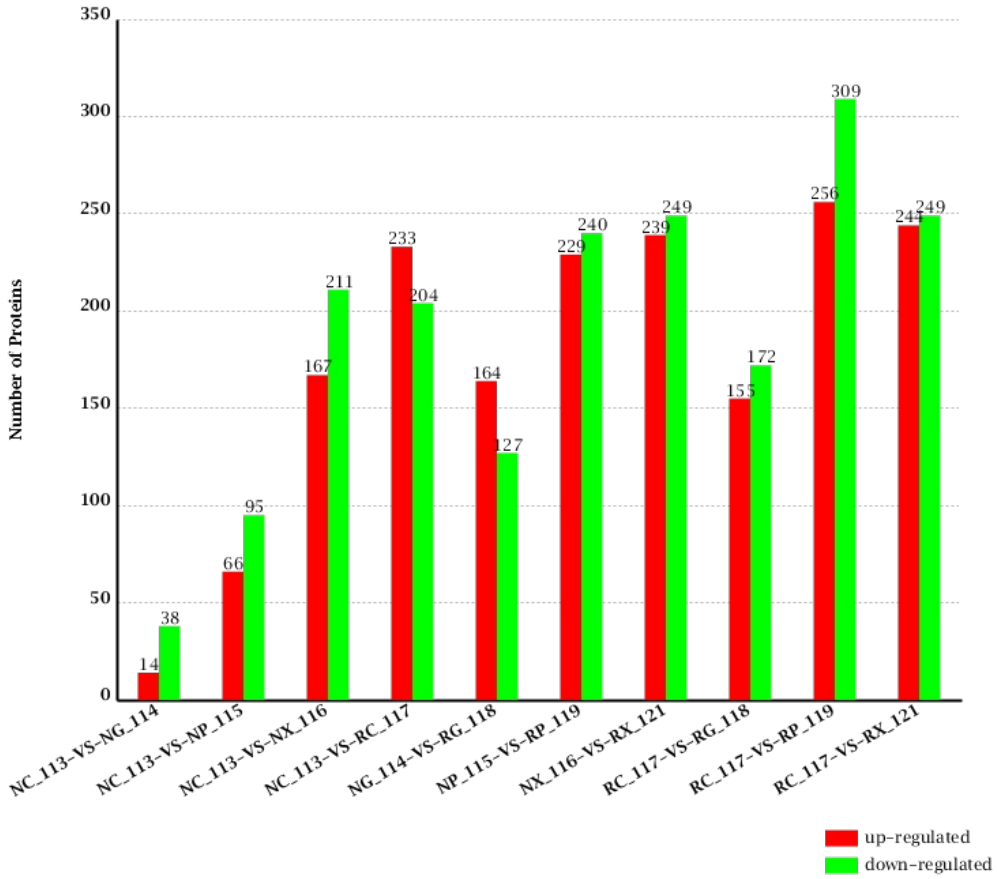

Fig4-7 Analysis of Differentially Expressed Protein X-axis: names of the samples; Y-axis: the number of differentially expressed protein. Red column: The number of up-regulation protein; Green column: The number of down-regulation protein.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Image/significant.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_2/Image/significant.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_3/Image/significant.png

F13FTSEUHT0743-004_iTRAQ_20140304/combined/Image/significant.png

4.6.4 Protein Abundance Distribution

In the relative quantification, if the protein quantification in 2 samples don't change obviously, its ratio of the protein abudance is close to 1. when the ratio of the protein abudance achieved more the time and its p-value<0.05, this protein is regareded differentially expressed protein.The following figure shows the protein ratio [log(protein ratio),with base=2] of each differentially expressed protein. Up-regulation protein is located right of zero of X-axis, down-regulation protein is located left of zero of X-axis.

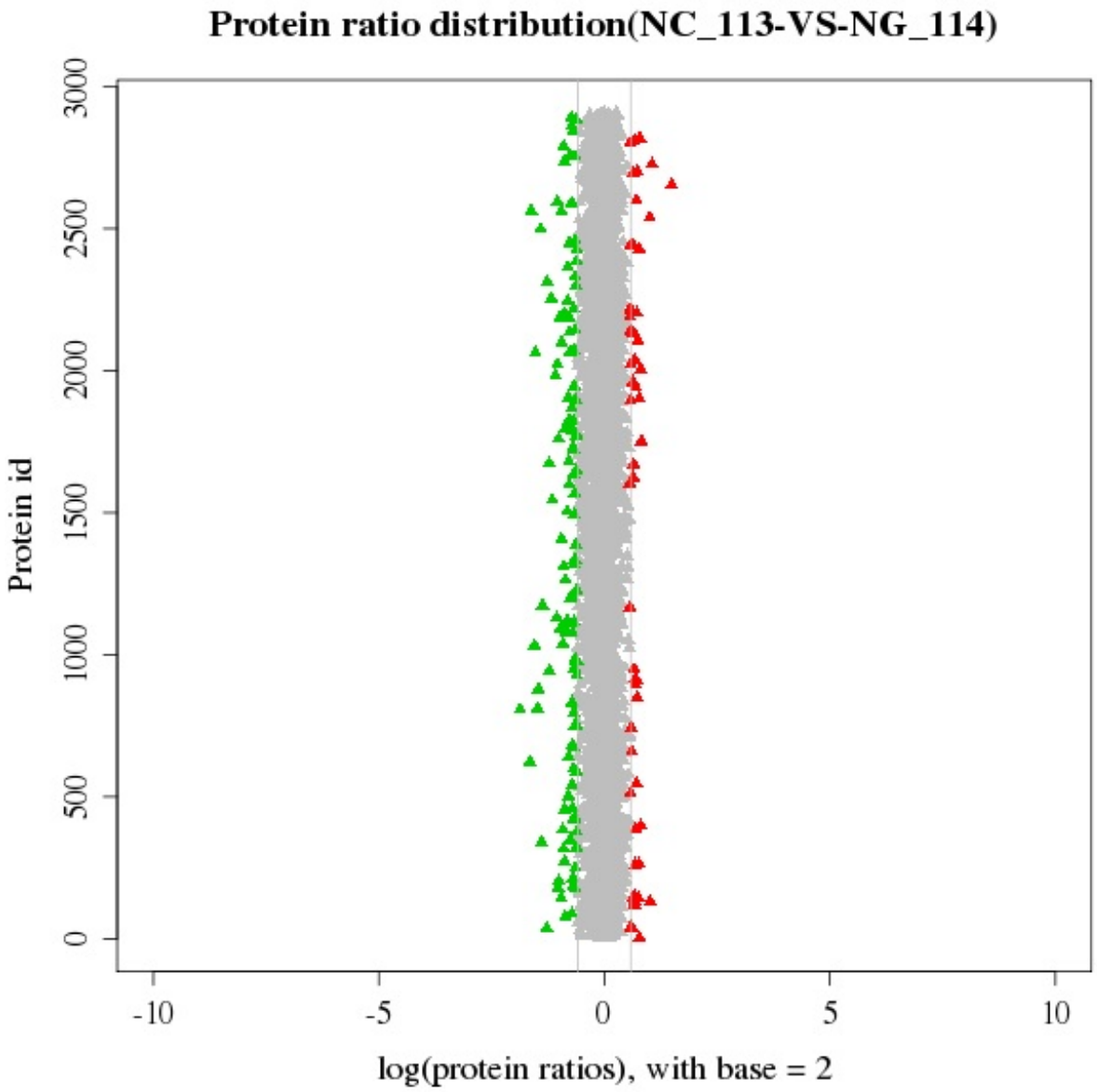

Fig4-8 Protein Abundance Distribution This picture shows the distribution of the fold change of all the quantified proteins. The X-axis shows protein ratio[log(protein ratio),with base=2]. when its protein ratio is above 0, it means it's up-regulation. when it's under 0, it's down-regulated. Mark the dot which shows the large fold change with red and green(red means up-regulated, green means down-regulated). This red and green dots may be the potential differentially expressed Protein, whether it's the accurate differentially expressed Protein should be identified by statistics.

Outcome Document:

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NG_114.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NG_114_down_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NG_114_up_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NP_115.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NP_115_down_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NP_115_up_annot.xls

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NX_116.png

F13FTSEUHT0743-004_iTRAQ_20140304/repeat_1/Quantitation/NC_113-VS-NX_116_down_annot.xls

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4.7 Replicate Analyses

The source of variation differs from one experiment to another, and it is a function of time, manpower, instrument,

subject, subject condition, and preparation process, among others. These sources of variation (measured experimentally or technically) must be minimized or identified. By definition,the variation is a measure of its dispersion, indicating how its possible values are spread around the expected value. In the biosciences, this can be measured as three different forms: technical, experimental, and biological.

[source]:Chee SG, Poh KC, Trong KP, Wright PC. Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ) Journal of Proteome Research. 2007;6(2):821–827

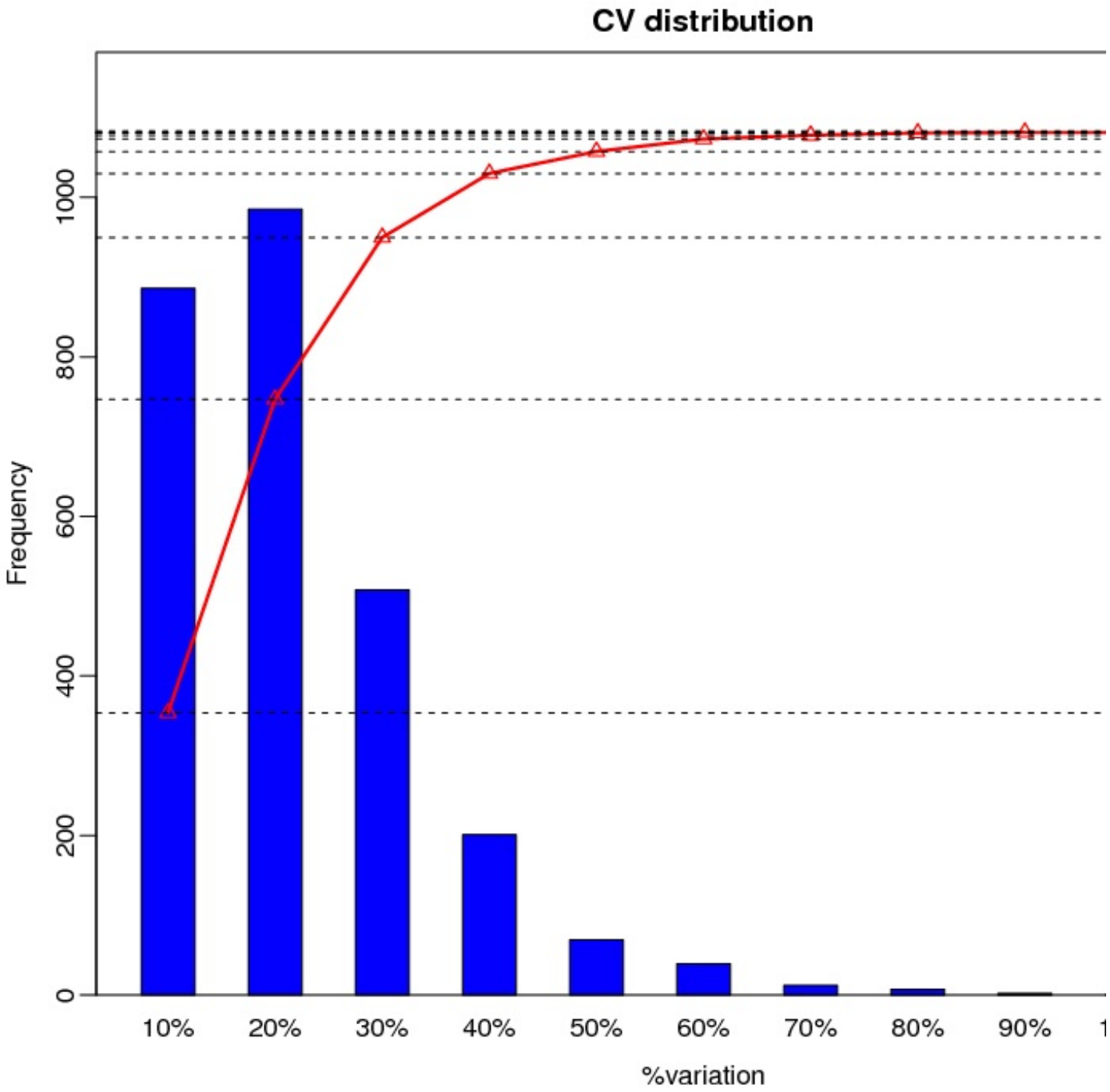

Fig4-9 Replicate Analyses Fig X-axis is coefficient of variation (CV) between the protein ratio(log2) of the 3 repliates.Y-axis(left) is the cumulative frequency that proteins happen at a certain CV value.Y-axis(right) is the cumulative percentage that protein at a certain CV value comprise quantified protein amout.

Outcome Document:

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5 ADVANCED BIOINFORMATICS ANALYSIS

5.1 GO Annotation

The Gene Ontology, or GO, is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species.More specifically, the project aims to:

1. Maintain and develop its controlled vocabulary of gene and gene product attributes;

2. Annotate genes and gene products, and assimilate and disseminate annotation data;

3. Provide tools for easy access to all aspects of the data provided by the project.

The ontology covers three domains:

1. Cellullar component A cellular component is just that, a component of a cell, but with the proviso that it is part of some larger object; this may be an anatomical structure (e.g. rough endoplasmic reticulum or nucleus) or a gene product group (e.g. ribosome, proteasome or a protein dimer).

2. Molecular function Molecular function describes activities, such as catalytic or binding activities, that occur at the molecular level. GO molecular function terms represent activities rather than the entities(molecules or complexes) that perform the actions, and do not specify where or when, or in what context, the action takes place.

3. Biological process A biological process is series of events accomplished by one or more ordered assemblies of molecular functions. It can be difficult to distinguish between a biological process and a molecular function, but the general rule is that a process must have more than one distinct steps.

[source-1]:http://en.wikipedia.org/wiki/Gene_Ontology

[source-2]:http://www.geneontology.org/GO.doc.shtml#cellular_component

We make GO analysis based all identified proteins in this part. The results are included in protein2go and go2protein files.

protein2go: show the GO ID for every protein.

go2protein: list all matched protein ID and protein number for every GO term.

Meanwhile, a pie chart for every ontology shows the match ratio.

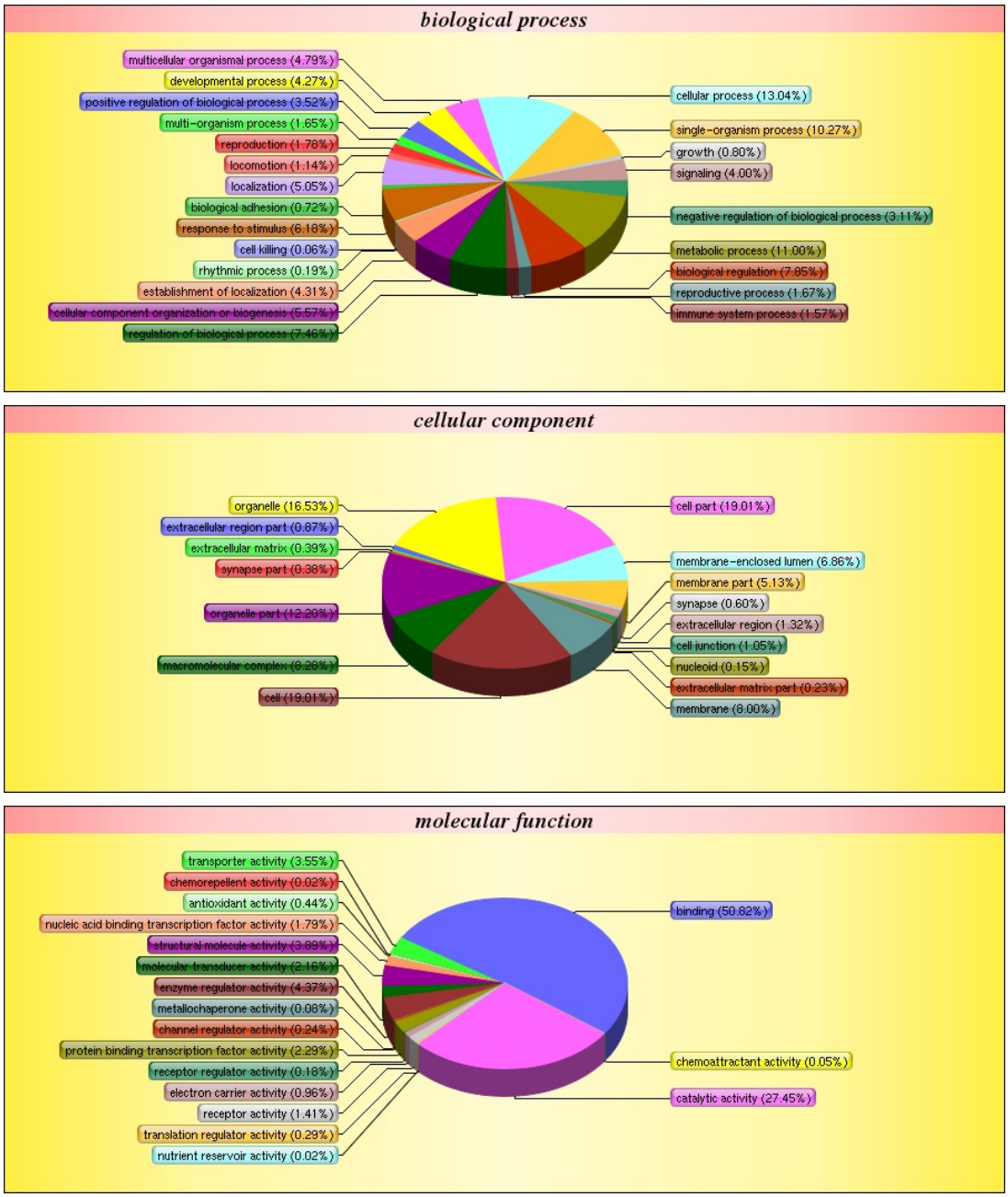

Fig5-1 Pie chart of the Gene Ontology Analysis The pie chart shows the distribution of corresponding GO terms.Different colors stand for different GO terms and the area show the GO term's percentage of all proteins.

Outcome Document:

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5.2 COG Annotation

Clusters of Orthologous Groups of proteins (COGs) were delineated by comparing protein sequences encoded in complete genomes, representing major phylogenetic lineages. Each COG consists of individual proteins or groups of paralogs from at least 3 lineages and thus corresponds to an ancient conserved domain. Each COG consists of individual orthologous proteins or orthologous sets of paralogs from at least three lineages. Orthologs typically have the same function, allowing transfer of functional information from one member to an entire COG. This relation automatically yields a number of functional predictions for poorly characterized genomes. The COGs comprise a framework for functional and evolutionary genome analysis.

[source-1]:http://www.ncbi.nlm.nih.gov/pubmed/9381173?dopt=Abstract

[source-2]:http://www.ncbi.nlm.nih.gov/pubmed/12969510

[source-3]:http://www.ncbi.nlm.nih.gov/COG/

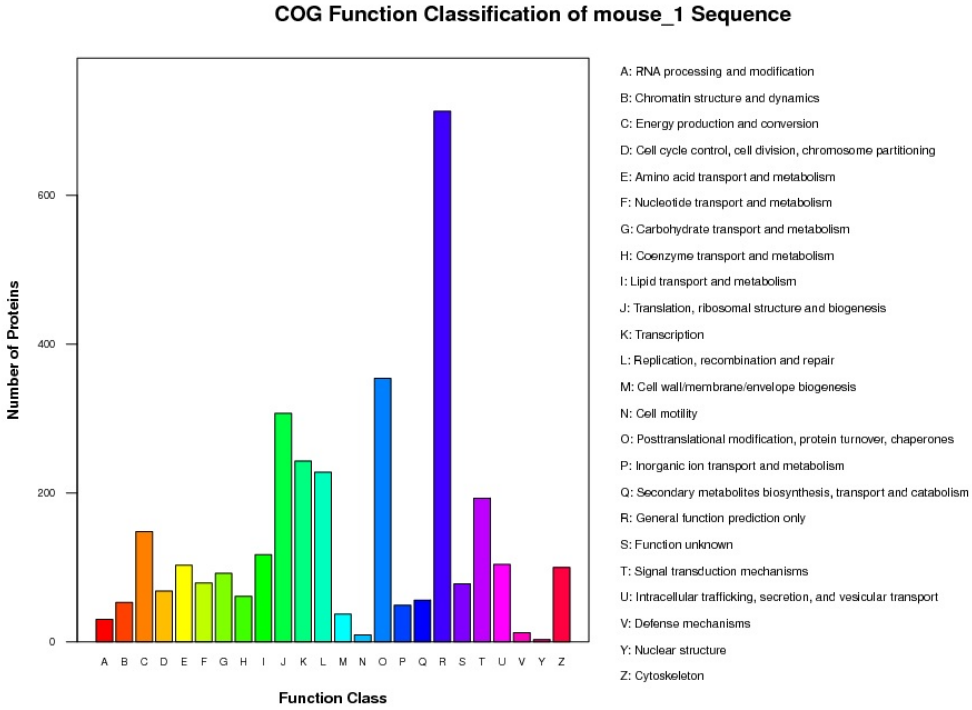

Fig5-2 Histogram of the GOG Analysis X-axis displays the COG term, Y-axis displays the corresponding protein count illustrating the protein number ofdifferent function.

Outcome Document:

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F13FTSEUHT0743-004_iTRAQ_20140304/combined/COG/all_protein.protein2cog.xls

5.3 Pathway Annotation

KEGG PATHWAY is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks for:

0. Global Map

1. Metabolism

Carbohydrate Energy Lipid Nucleotide Amino acid Other amino acid Glycan Cofactor/vitamin Terpenoid/PK Other secondary met abolite Xenobiotics Reaction module Chemical structure.

2. Genetic Information Processing

3. Environmental Information Processing

4. Cellular Processes

5. Organismal Systems

6. Human Diseases

and also on the structure relationships (KEGG drug structure maps) in:

7. Drug Development

[source]:http://www.genome.jp/kegg/pathway.html

Outcome Document:

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5.4 GO Enrichment Analysis

GO enrichment analysis shows the GO terms which the differentially expressed proteins enriched in all identified proteins. It represents the important or typical biology functions in the project. We define a differentially expressed protein to be significantly regulated if the p-value is less than 0.05.In GO enrichment analysis, we use hyper geometric test to get the target GO terms. The formula is as follows:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

N: is the population size, the number of all identified proteins which matched to GO terms. n: is the number of draws. The number of differentially expressed proteins. M: is the number of success states in the population, the number of proteins which matched a certain GO term. m: is the number of successes, the number of differentially expressed proteins which matched a certain GO term. If the p-value is less than 0.05, the GO term is significant enrichment in differentially expressed proteins.

[source]:http://en.wikipedia.org/wiki/Hypergeometric_distribution

Result Table

Terms from the Component Ontology with p-value as good or better than 1

Gene Ontology term	Cluster frequency	Protein frequency of use	P-value
ribonucleoprotein complex	12 out of 36 genes, 33.3%	90 out of 766 genes, 11.7%	0.0003887879
intracellular	30 out of 36 genes, 83.3%	511 out of 766 genes, 66.7%	0.01947108
chromatin	2 out of 36 genes, 5.6%	6 out of 766 genes, 0.8%	0.02860762
intracellular part	29 out of 36 genes, 80.6%	503 out of 766 genes, 65.7%	0.03624558
macromolecular complex	14 out of 36 genes, 38.9%	197 out of 766 genes, 25.7%	0.05277838
cell	32 out of 36 genes, 88.9%	592 out of 766 genes, 77.3%	0.05989476
cell part	32 out of 36 genes, 88.9%	592 out of 766 genes, 77.3%	0.05989476
non-membrane-bounded organelle	5 out of 36 genes, 13.9%	47 out of 766 genes, 6.1%	0.06258606
intracellular non-membrane-bounded organelle	5 out of 36 genes, 13.9%	47 out of 766 genes, 6.1%	0.06258606
endoplasmic reticulum	2 out of 36 genes, 5.6%	9 out of 766 genes, 1.2%	0.06281128
chromosomal part	2 out of 36 genes, 5.6%	9 out of 766 genes, 1.2%	0.06281128
chromosome	2 out of 36 genes, 5.6%	11 out of 766 genes, 1.4%	0.09047561
cytoplasmic part	5 out of 36 genes, 13.9%	80 out of 766 genes, 10.4%	0.3197551
cytoplasm	5 out of 36 genes, 13.9%	81 out of 766 genes, 10.6%	0.3295799

Fig5-3 Set an example for Differentially Expressed protein GO

Enrichment Analysis This picture is the screenshot of GO Enrichment Analysis.Cluster frequency mean the ratio of Annotation the same Gene Onltolgy term between all of differentially Expressed protein and all of the protein.

Outcome Document:

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5.5 Pathway Enrichment Analysis

The method in Pathway enrichment analysis is same as GO enrichment analysis.Using these dataset, we can define the main or important pathways of this biological process depending on other knowledge about it.

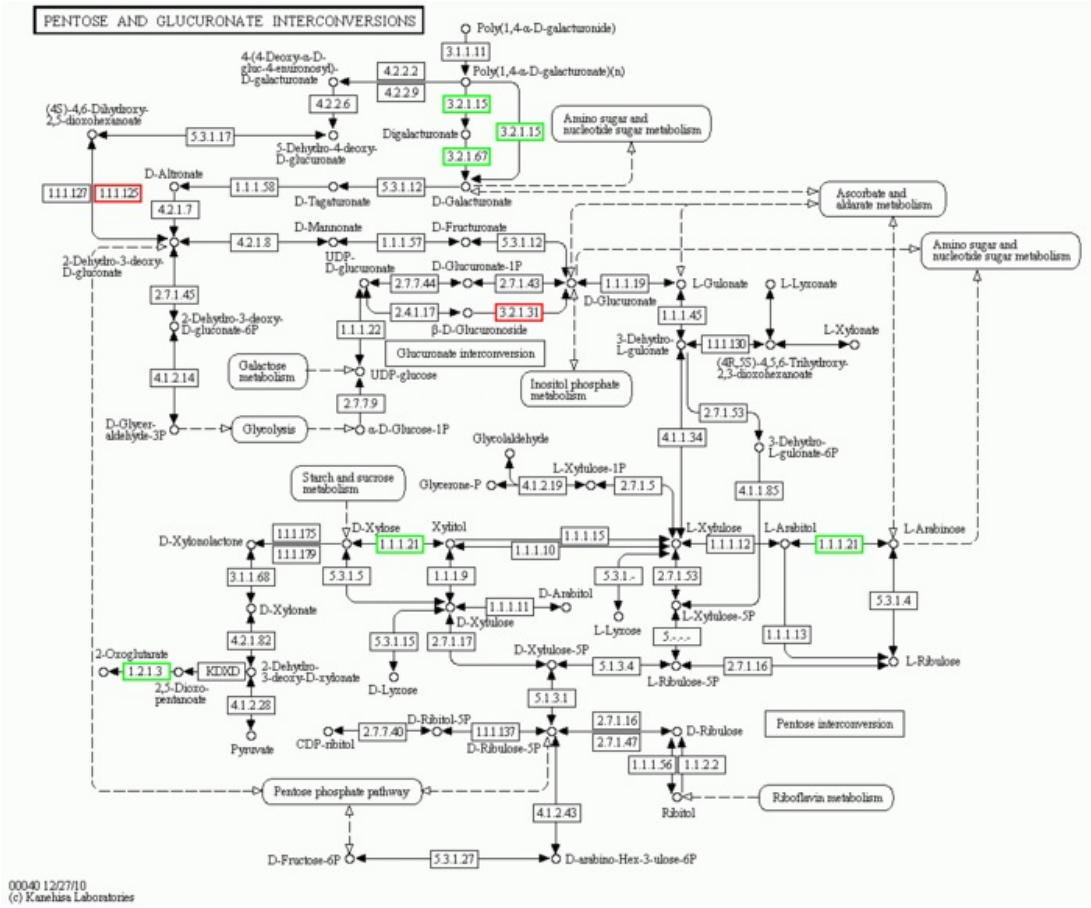

Fig5-4 Set an example for Pathway Enrichment Analysis This picture is a screenshot.Red colour:up-regulation protein.Green colour:down-regulation protein

Outcome Document:

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NG_114_Pathway_enrichment/NC_113-VS-NG_114.htm

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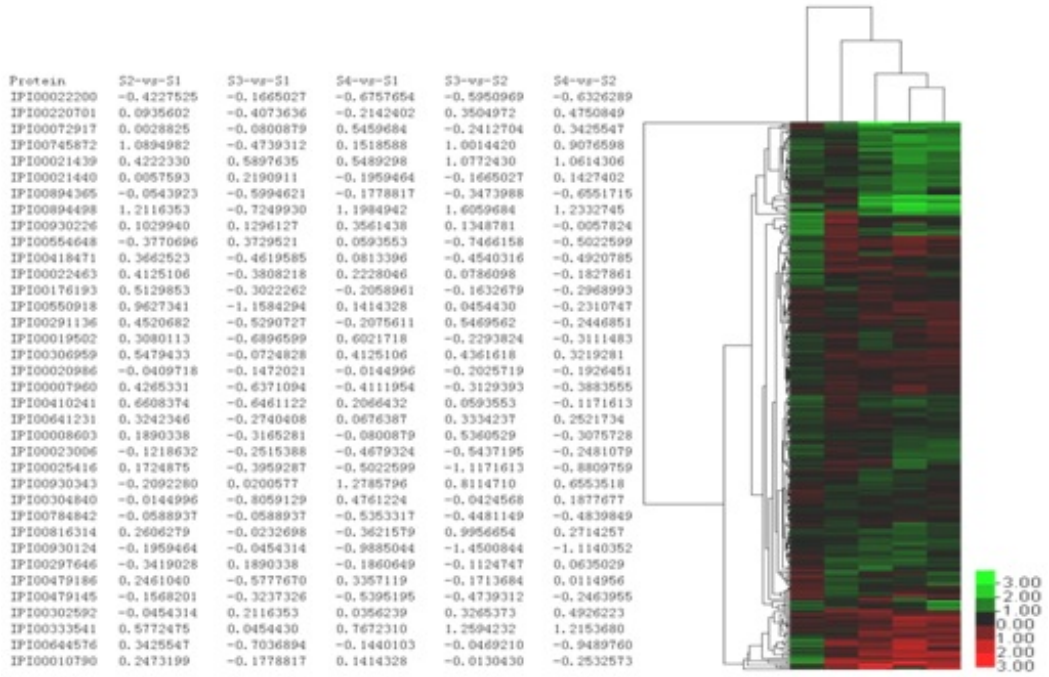
5.6 Cluster Analysis of Genes Expression Profiles

Cluster analysis is the task of grouping a set of objects in such a way that objects in the same group (called cluster) are more similar (in some sense or another) to each other than to those in other groups (clusters). It is a main task of exploratory data mining, and a common technique for statistical data analysis used in bioinformatics.

Cluster analysis itself is not one specific algorithm, but the general task to be solved. It can be achieved by various algorithms that differ significantly in their notion of what constitutes a cluster and how to efficiently find them. Popular notions of clusters include groups with small distances among the cluster members, dense areas of the data space, intervals or particular statistical distributions. Clustering can therefore be formulated as amulti-objective optimization problem.

We analyse the proteins expression patterns in different sample groups by cluster analysis using Euclidean distance and hierarchical algorithm.

[source]:http://en.wikipedia.org/wiki/Cluster_analysis



Protein	S2-vs-S1	S3-vs-S1	S4-vs-S1	S3-vs-S2	S4-vs-S2
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IPI00220701	0.0935602	-0.4073636	-0.2142402	0.3504972	0.4750849
IPI00072917	0.0028825	-0.0800879	0.5459684	-0.2412704	0.3425547
IPI00745872	1.0894982	-0.4739312	0.1518588	1.0014420	0.9076598
IPI00021439	0.4222330	0.5897635	0.5489298	1.0772430	1.0614306
IPI00021440	0.0057593	0.2190911	-0.1959464	-0.1665027	0.1427402
IPI00894365	-0.0543923	-0.5994621	-0.1778817	-0.3473988	-0.6551715
IPI00894498	1.2116353	-0.7249930	1.1984942	1.6059684	1.2332745
IPI00930226	0.1029940	0.1296127	0.3561438	0.1348781	-0.0057824
IPI00554648	-0.3770696	0.3729521	0.0593553	-0.7466158	-0.5022599
IPI00418471	0.3662523	-0.4619585	0.0813396	-0.4540316	-0.4920785
IPI00022463	0.4125106	-0.3808218	0.2228046	0.0786098	-0.1827861
IPI00176193	0.5129853	-0.3022262	-0.2058961	-0.1632679	-0.2968993
IPI00550918	0.9627341	-1.1584294	0.1414328	0.0454430	-0.2310747
IPI00291136	0.4520682	-0.5290727	-0.2075611	0.5469562	-0.2446851
IPI00019502	0.3080113	-0.6896599	0.6021718	-0.2293824	-0.3111483
IPI00306959	0.5479433	-0.0724828	0.4125106	0.4361618	0.3219281
IPI00020986	-0.0409718	-0.1472021	-0.0144996	-0.2025719	-0.1926451
IPI00007960	0.4265331	-0.6371094	-0.4111954	-0.3129393	-0.3883555
IPI00410241	0.6608374	-0.6461122	0.2066432	0.0593553	-0.1171613
IPI00641231	0.3242346	-0.2740408	0.0676387	0.3334237	0.2521734
IPI00008603	0.1890338	-0.3165281	-0.0800879	0.5360529	-0.3075728
IPI00023006	-0.1218632	-0.2515388	-0.4679324	-0.5437195	-0.2481079
IPI00025416	0.1724875	-0.3959287	-0.5022599	-1.1171613	-0.8809759
IPI00930343	-0.2092280	0.0200577	1.2785796	0.8114710	0.6553518
IPI00304840	-0.0144996	-0.8059129	0.4761224	-0.0424568	0.1877677
IPI00784842	-0.0588937	-0.0588937	-0.5353317	-0.4481149	-0.4839849
IPI00816314	0.2606279	-0.0232698	-0.3621579	0.9956654	0.2714257
IPI00930124	-0.1959464	-0.0454314	-0.9885044	-1.4500844	-1.1140352
IPI00297646	-0.3419028	0.1890338	-0.1860649	-0.1124747	0.0635029
IPI00479186	0.2461040	-0.5777670	0.3357119	-0.1713684	0.0114956
IPI00479145	-0.1568201	-0.3237326	-0.5395195	-0.4739312	-0.2463955
IPI00302592	-0.0454314	0.2116353	0.0356239	0.3265373	0.4926223
IPI00333541	0.5772475	0.0454430	0.7672310	1.2594232	1.2153680
IPI00644576	0.3425547	-0.7036894	-0.1440103	-0.0469210	-0.9489760
IPI00010790	0.2473199	-0.1778817	0.1414328	-0.0130430	-0.2532573

3.00
2.00
1.00
0.00
1.00
2.00
3.00

Fig5-5 Cluster Analysis of Genes Expression Profiles cluster analysis of genes expression profiles Normalized proteins data from different sample groups are subjected to Cluster3.0 software and visualized using Java TreeView.Quantified proteins are grouped based on sample groups. Green indicates down-regulation, red indicates up-regulation, Gray indicates no detectable expression.

Outcome Document:

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6 DATA DOWNLOADING

6.1 FTP address

Host: http://cdts.genomics.org.cn/

ID:xxx

6.2 File Decompress

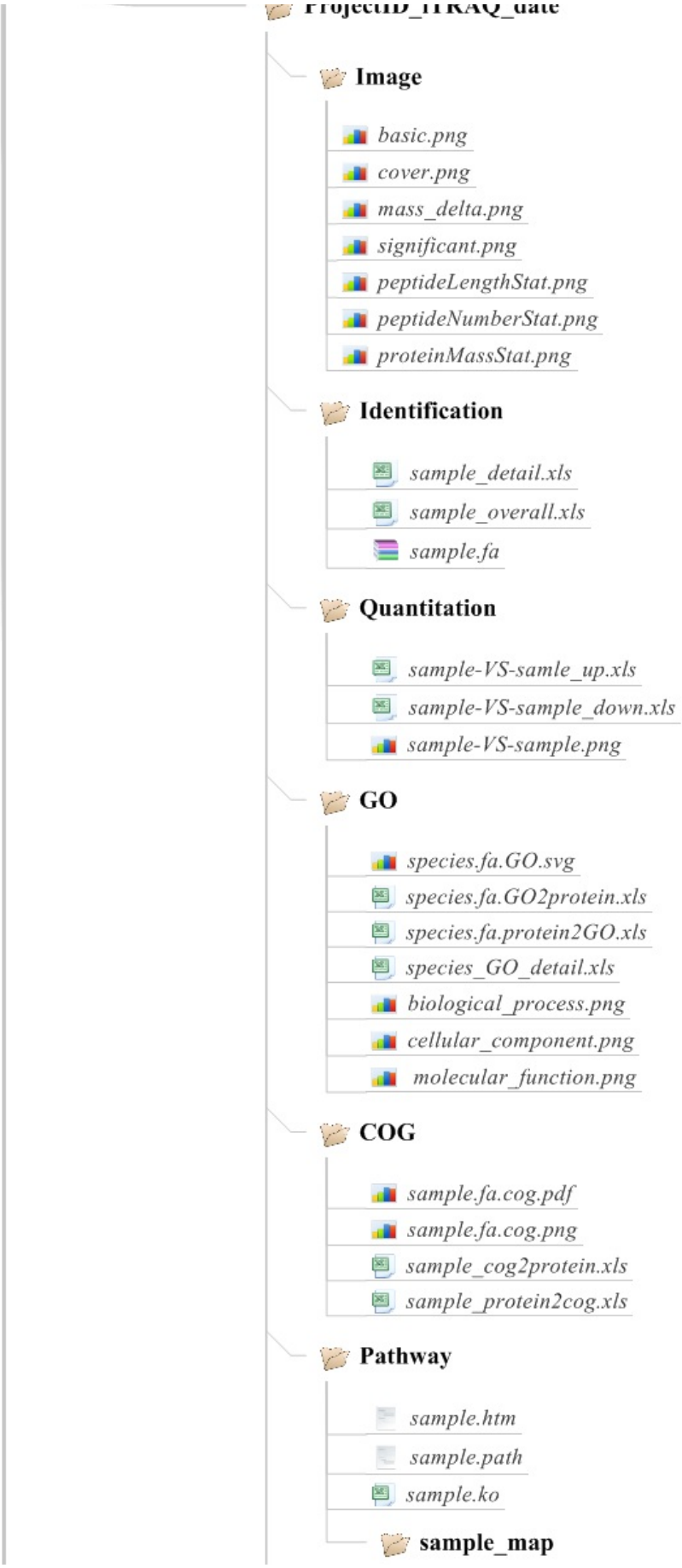
All files are compressed into *.tar.gz format. You can decompression them by this way:

Unix/Linux user： tar –zxvf *.tar.gz or gzip –d *.gz.

Windows user：winRAR tool

Mac user: tar –zxvf *.tar.gz.

6.3 FTP Directory Structure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cover.png
mass_delta.png
significant.png
peptideLengthStat.png
peptideNumberStat.png
proteinMassStat.png
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sample_overall.xls
sample.fa
Quantitation
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sample-VS-sample_down.xls
sample-VS-sample.png
GO
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species.fa.GO2protein.xls
species.fa.protein2GO.xls
species_GO_detail.xls
biological_process.png
cellular_component.png
molecular_function.png
COG
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sample_cog2protein.xls
sample_protein2cog.xls
Pathway
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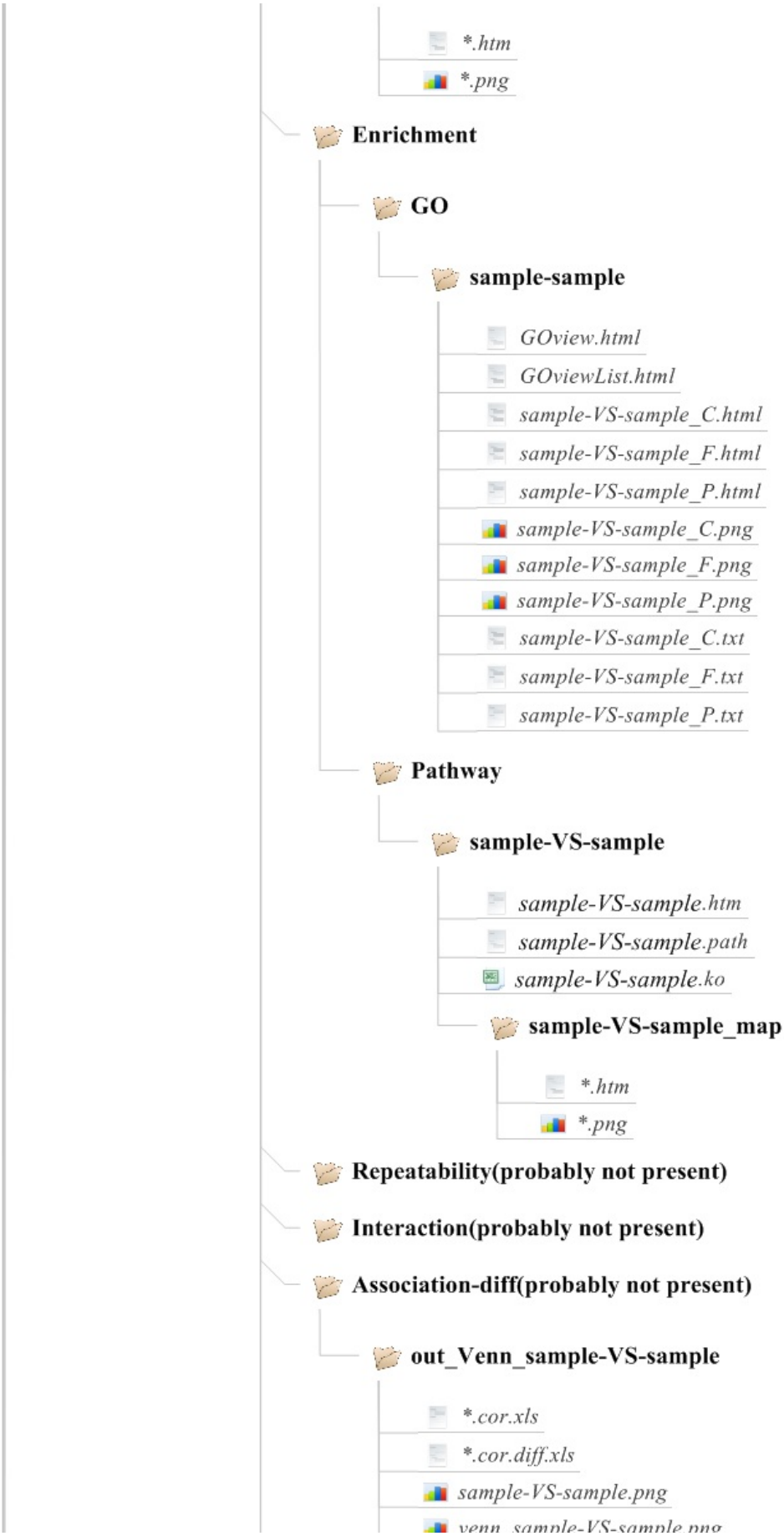
sample.path
sample.ko
sample_map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Enrichment
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GOview.html
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sample-VS-sample_F.html
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sample-VS-sample_C.png
sample-VS-sample_F.png
sample-VS-sample_P.png
sample-VS-sample_C.txt
sample-VS-sample_F.txt
sample-VS-sample_P.txt
Pathway
sample-VS-sample
sample-VS-sample.htm
sample-VS-sample.path
sample-VS-sample.ko
sample-VS-sample_map
*.htm
*.png
Repeatability(probably not present)
Interaction(probably not present)
Association-diff(probably not present)
out_Venn_sample-VS-sample
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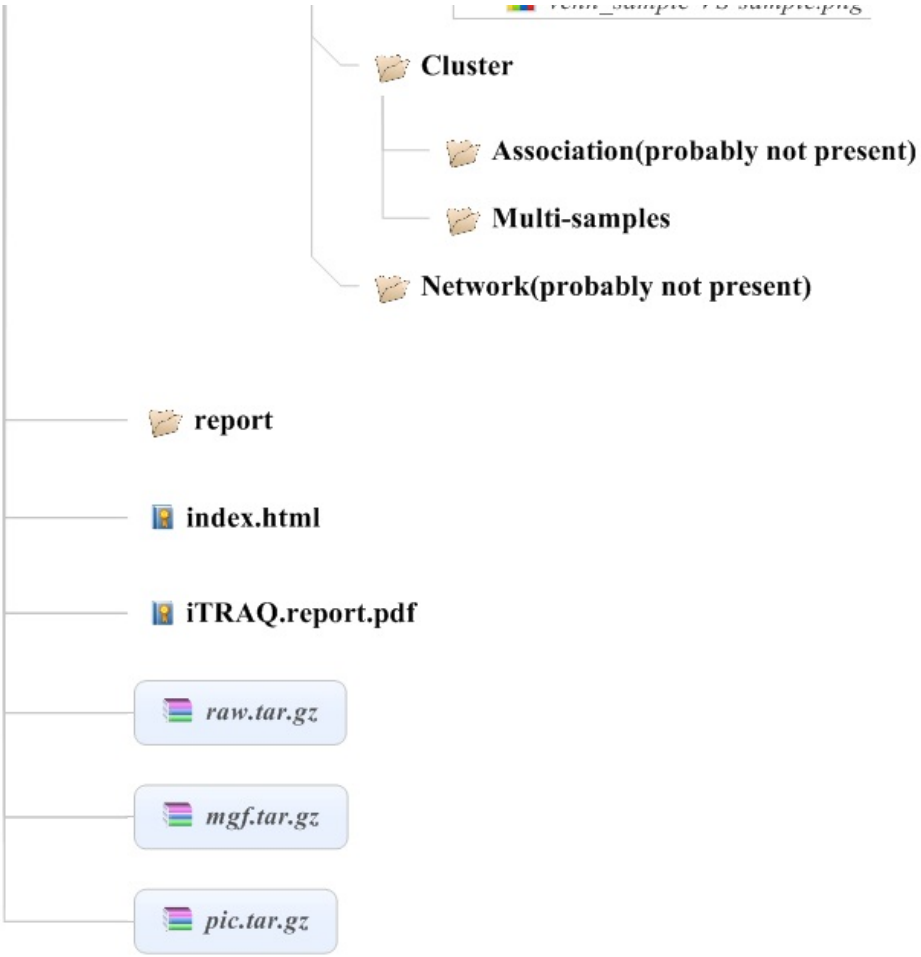
*.cor.diff.xls
sample-VS-sample.png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Multi-samples
Network(probably not present)
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