APPENDICES

Appendix 1: ACE inhibitory activity

Table 1.1 : ACE inhibitory activity of Captopril

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Captopril 1 x 10^{-6} mg/mL	96.10	95.93	95.76	95.93	0.17	0.10
Captopril 1 x 10^{-8} mg/mL	88.64	89.92	85.51	88.02	2.27	1.31
Captopril 1 x 10^{-9} mg/mL	38.48	37.88	42.29	39.55	2.39	1.38
Captopril 1 x 10^{-10} mg/mL	12.80	6.36	5.34	8.17	4.05	2.34
Captopril 1 x 10^{-11} mg/mL	0	0	0	0	0	0

Table 1.2 : ACE inhibitory activity of mycelia crude water extract

Sample	Inhibition rate (%)		Mean (%)	SD	SEM
	R1	R2			
Mycelia 10 mg/mL	95.16	95.84	95.50	0.48	0.34
Mycelia 5 mg/mL	88.28	89.18	88.73	0.64	0.45
Mycelia 2.5 mg/mL	86.16	70.88	78.52	10.81	7.64
Mycelia 1.25 mg/mL	59.76	60.82	60.29	0.75	0.53
Mycelia 0.625 mg/mL	43.12	39.71	41.42	2.41	1.70

Table 1.3 : ACE inhibitory activity of broth crude water extract

Sample	Inhibition rate (%)		Mean (%)	SD	SEM
	R1	R2			
Broth 60 mg/mL	83.28	83.13	83.21	0.11	0.08
Broth 50 mg/mL	81.92	80.26	81.09	1.17	0.83
Broth 40 mg/mL	74.43	74.13	74.28	0.21	0.15
Broth 30 mg/mL	71.33	71.71	71.52	0.27	0.19
Broth 20 mg/mL	59.68	63.09	61.39	2.41	1.70
Broth 10 mg/mL	47.20	46.45	46.83	0.53	0.38
Broth 1 mg/mL	9.00	7.87	8.44	0.80	0.57

Table 1.4 : ACE inhibitory activity of protein fraction A

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Fraction A 400 µg/mL	84.30	85.13	85.52	84.98	0.62	0.36
Fraction A 200 µg/mL	70.64	72.20	66.93	69.92	2.71	1.57
Fraction A 100 µg/mL	43.62	45.54	46.13	45.10	1.31	0.76
Fraction A 50 µg/mL	25.91	27.31	25.61	26.28	0.91	0.53
Fraction A 25 µg/mL	7.90	12.47	11.73	10.70	2.45	1.41

Table 1.5 : ACE inhibitory activity of protein fraction B

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Fraction B 400 µg/mL	85.29	83.3	83.24	83.94	1.17	0.68
Fraction B 200 µg/mL	68.37	69.09	68.15	68.54	0.49	0.28
Fraction B 100 µg/mL	43.32	45.90	42.95	44.06	1.61	0.93
Fraction B 50 µg/mL	27.68	31.37	33.14	30.73	2.79	1.61
Fraction B 25 µg/mL	17.86	39.19	15.65	24.23	1.56	0.90

Table 1.6 : ACE inhibitory activity of protein fraction C

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Fraction C 400 µg/mL	83.41	82.24	79.69	81.78	1.90	1.10
Fraction C 200 µg/mL	64.10	67.040	67.09	66.08	1.71	0.99
Fraction C 100 µg/mL	48.78	48.27	48.04	48.36	0.38	0.22
Fraction C 50 µg/mL	24.80	26.72	24.50	25.34	1.21	0.70
Fraction C 25 µg/mL	15.42	17.79	17.71	17.75	1.35	0.78

Table 1.7 : ACE inhibitory activity of protein fraction D

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Fraction D 400 µg/mL	82.19	77.97	79.86	80.01	2.11	1.22
Fraction D 200 µg/mL	65.48	61.93	57.77	61.73	3.86	2.23
Fraction D 100 µg/mL	38.67	38.16	36.61	37.81	1.07	0.62
Fraction D 50 µg/mL	29.89	23.62	30.92	28.14	3.95	2.28
Fraction D 25 µg/mL	11.22	16.90	22.80	16.97	5.79	3.34

Table 1.8 : ACE inhibitory activity of protein fraction E

Sample	Inhibition rate (%)			Mean %	SD	SEM
	R1	R2	R3			
Fraction E 400 µg/mL	65.87	62.93	62.88	63.89	1.71	0.99
Fraction E 200 µg/mL	49.33	41.23	46.62	45.73	4.12	2.38
Fraction E 100 µg/mL	20.74	21.92	24.35	22.34	1.84	1.06
Fraction E 50 µg/mL	14.10	13.38	16.31	14.60	1.53	0.88
Fraction E 25 µg/mL	11.44	11.00	11.37	11.27	0.24	0.14

Table 1.9 : ACE inhibitory activity of HPLC peaks at 25 µg/mL

Sample	Inhibition rate (%)		Mean %	SD	SEM
	R1	R2			
A1	32.69	28.39	30.54	3.04	2.15
A2	13.15	19.21	16.18	4.29	3.03
A3	14.39	13.77	14.08	0.44	0.31
A4	18.58	15.98	17.28	1.84	1.30
A5	49.58	43.40	46.49	4.37	3.09
A6	33.09	27.65	30.37	3.85	2.72
C1	37.90	39.09	38.50	0.84	0.59
C2	28.78	46.23	37.51	12.34	8.73
C3	68.67	67.20	67.94	1.04	0.74
C4	57.51	58.41	57.96	0.64	0.45
C5	62.44	57.68	60.06	3.37	2.38
C6	26.46	25.27	25.87	0.84	0.59
C7	47.14	45.67	46.41	1.04	0.74

Table 1.10 : ACE inhibitory activity of HPLC peak C3

Sample	Inhibition percentage			Mean %	SD	SEM
	R1	R2	R3			
C3 50 µg/mL	83.81	83.45	81.75	83.00	1.10	0.64
C3 25 µg/mL	70.22	71.32	69.01	70.18	1.16	0.67
C3 12.5 µg/mL	56.16	56.4	55.19	55.92	0.64	0.37
C3 6.25 µg/mL	43.06	39.12	40.39	40.86	2.01	1.16
C3 3.125 µg/mL	33.05	33.48	32.32	32.95	0.59	0.34

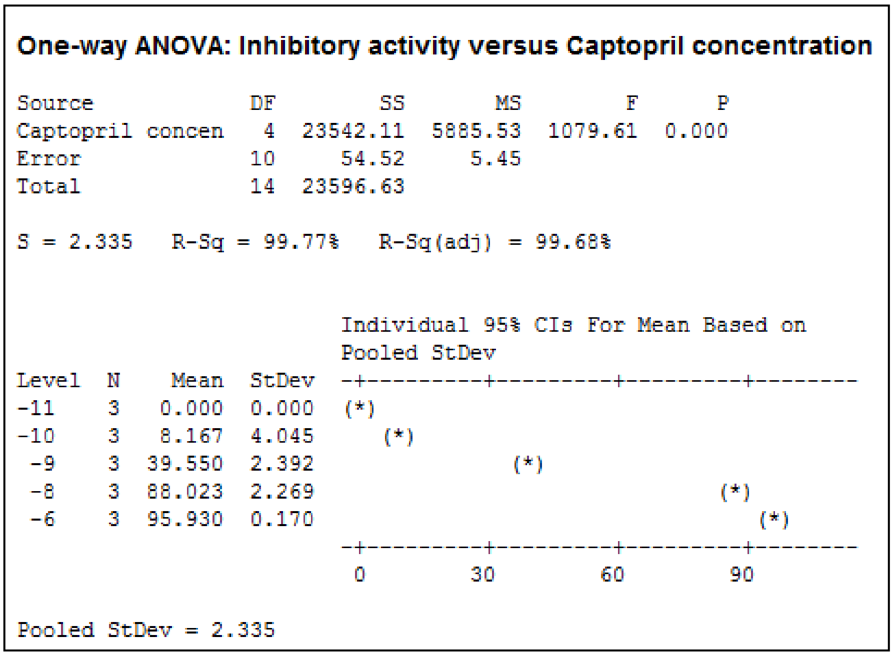
Table 1.11 : ACE inhibitory activity of HPLC peak C4

Sample	Inhibition percentage			Mean %	SD	SEM
	R1	R2	R3			
C4 50 µg/mL	76.05	73.50	75.38	74.98	1.32	0.76
C4 25 µg/mL	60.22	60.64	58.64	59.83	1.06	0.61
C4 12.5 µg/mL	45.79	45.36	37.48	42.88	4.68	2.70
C4 6.25 µg/mL	37.17	39.18	31.60	35.98	3.93	2.27
C4 3.125 µg/mL	28.26	30.38	26.32	28.32	2.03	1.17

Table 1.12 : ACE inhibitory activity of HPLC peak C5

Sample	Inhibition percentage			Mean %	SD	SEM
	R1	R2	R3			
C5 50 µg/mL	69.80	72.04	71.62	71.15	1.19	0.69
C5 25 µg/mL	62.10	60.52	57.61	60.08	2.28	1.32
C5 12.5 µg/mL	49.73	49.30	51.00	50.01	0.88	0.51
C5 6.25 µg/mL	24.08	21.35	38.69	28.04	9.32	5.38
C5 3.125 µg/mL	16.89	8.55	0	12.72	8.45	4.88

Appendix 2: One-way ANOVA



One-way ANOVA: Inhibitory activity versus Captopril concentration

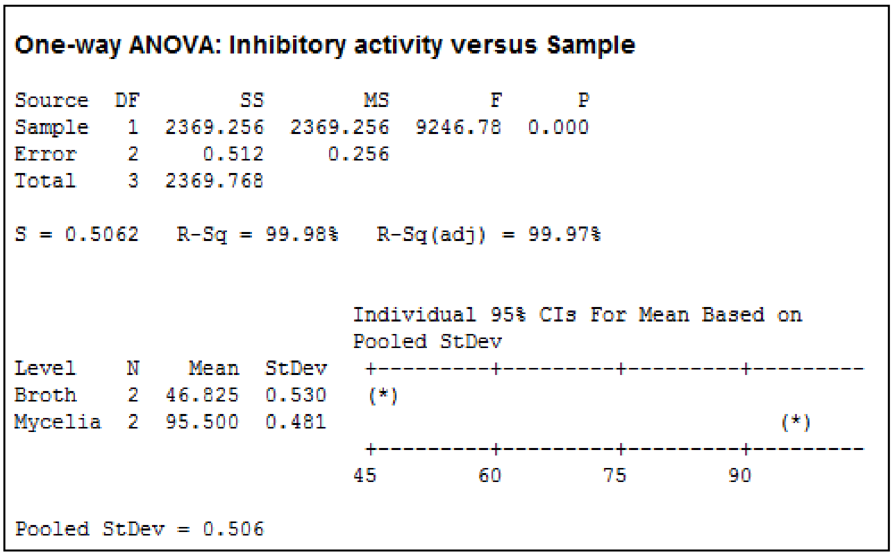
```
Source             DF        SS       MS        F      P
Captopril concen    4  23542.11  5885.53  1079.61  0.000
Error              10     54.52     5.45
Total              14  23596.63

S = 2.335   R-Sq = 99.77%   R-Sq(adj) = 99.68%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  -+---------+---------+---------+--------
-11    3   0.000  0.000  (*)
-10    3   8.167  4.045     (*)
 -9    3  39.550  2.392             (*)
 -8    3  88.023  2.269                             (*)
 -6    3  95.930  0.170                                (*)
                         -+---------+---------+---------+--------
                          0        30        60        90

Pooled StDev = 2.335
```

Figure 2.1 : One-way ANOVA test ACE inhibitory effect of Captopril at different concentrations



One-way ANOVA: Inhibitory activity versus Sample

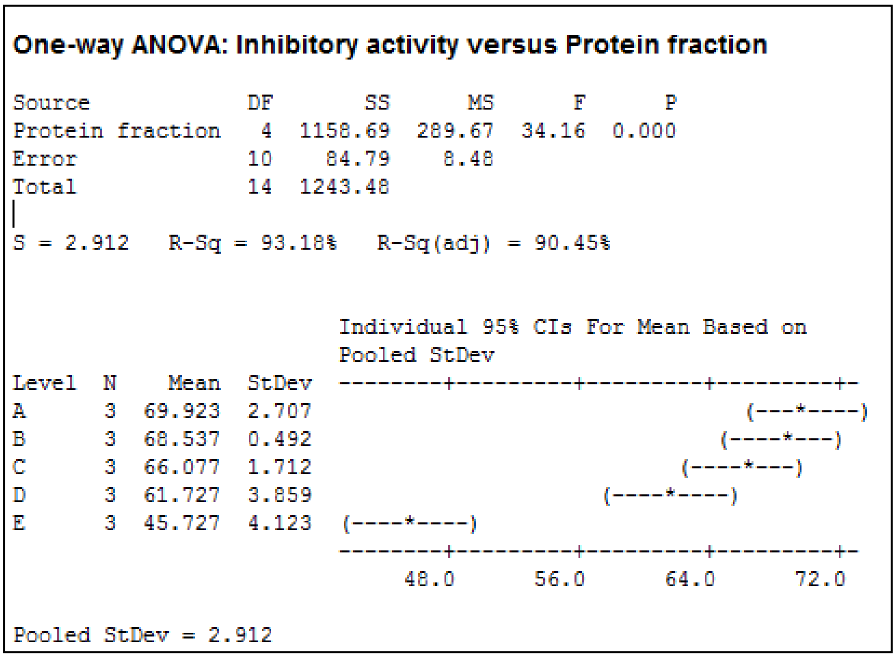
```
Source  DF        SS        MS        F      P
Sample   1  2369.256  2369.256  9246.78  0.000
Error    2     0.512     0.256
Total    3  2369.768

S = 0.5062   R-Sq = 99.98%   R-Sq(adj) = 99.97%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level     N    Mean  StDev  +---------+---------+---------+---------
Broth     2  46.825  0.530  (*)
Mycelia   2  95.500  0.481                                 (*)
                            +---------+---------+---------+---------
                           45        60        75        90

Pooled StDev = 0.506
```

Figure 2.2 : One-way ANOVA test for ACE inhibitory effect of 10 mg/mL of crude water extracts



One-way ANOVA: Inhibitory activity versus Protein fraction

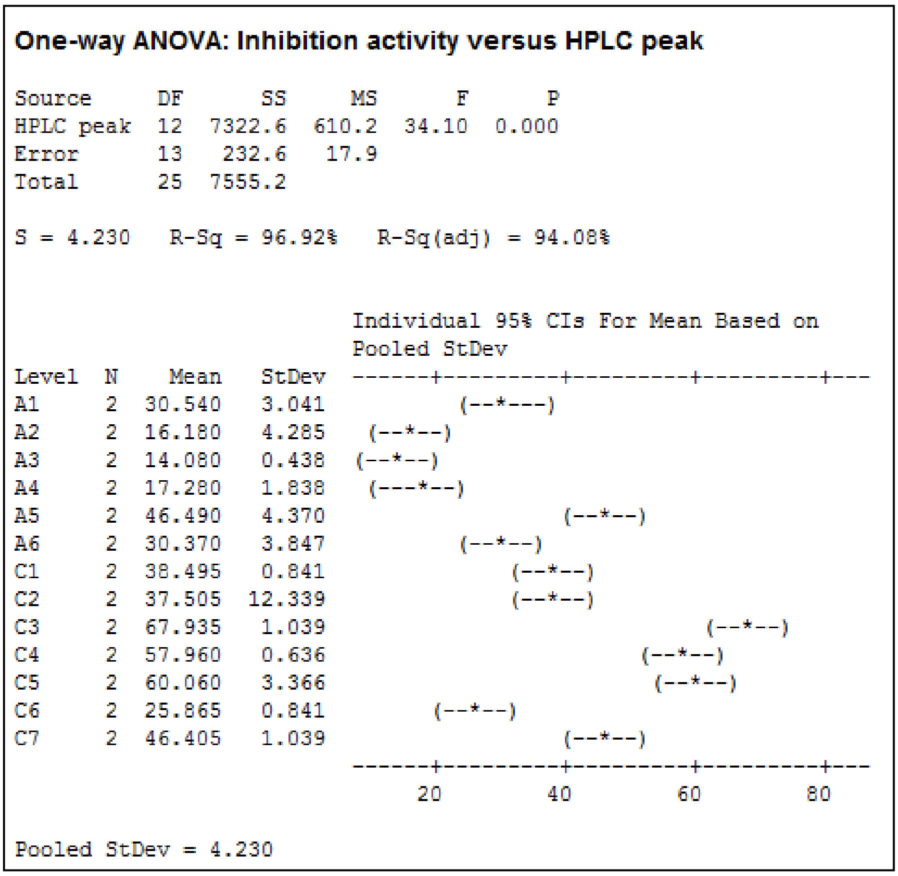
```
Source            DF       SS      MS      F      P
Protein fraction   4  1158.69  289.67  34.16  0.000
Error             10    84.79    8.48
Total             14  1243.48

S = 2.912   R-Sq = 93.18%   R-Sq(adj) = 90.45%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
A      3  69.923  2.707                                 (---*----)
B      3  68.537  0.492                               (----*---)
C      3  66.077  1.712                            (----*---)
D      3  61.727  3.859                      (----*----)
E      3  45.727  4.123  (----*----)
                         --------+---------+---------+---------+-
                              48.0      56.0      64.0      72.0

Pooled StDev = 2.912
```

Figure 2.3 : One-way ANOVA test for ACE inhibitory effect of 200 µg/mL of protein fractions



One-way ANOVA: Inhibition activity versus HPLC peak

```
Source     DF      SS     MS      F      P
HPLC peak  12  7322.6  610.2  34.10  0.000
Error      13   232.6   17.9
Total      25  7555.2

S = 4.230   R-Sq = 96.92%   R-Sq(adj) = 94.08%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
A1     2  30.540   3.041          (--*---)
A2     2  16.180   4.285   (--*--)
A3     2  14.080   0.438  (--*--)
A4     2  17.280   1.838   (---*--)
A5     2  46.490   4.370                  (--*--)
A6     2  30.370   3.847          (--*--)
C1     2  38.495   0.841              (--*--)
C2     2  37.505  12.339              (--*--)
C3     2  67.935   1.039                             (--*--)
C4     2  57.960   0.636                        (--*--)
C5     2  60.060   3.366                         (--*--)
C6     2  25.865   0.841        (--*--)
C7     2  46.405   1.039                  (--*--)
                          ------+---------+---------+---------+---
                               20        40        60        80

Pooled StDev = 4.230
```

Figure 2.4 : One-way ANOVA test for ACE inhibitory effect of 25 µg/mL of HPLC peaks

Appendix 3: Results from Mascot program

Mascot Search Results for C3-a

```
1.    MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
      Metallothionein (MT) - Neurospora crassa
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      2233.53   2232.52   2232.67    -0.15    2   - 26    0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
      No match to: 807.56, 832.65, 842.67, 842.67, 854.21, 856.68, 856.68, 864.66, 865.18, 865.18, 870.71, 870.71, 877.21,
      887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1045.77, 1060.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76,
      1107.76, 1109.71, 1141.76, 1157.81, 1157.81, 1165.81, 1179.83, 1179.83, 1193.84, 1201.84, 1201.84, 1234.91, 1234.91,
      1265.88, 1277.95, 1277.95, 1300.78, 1302.96, 1307.93, 1307.93, 1320.85, 1323.92, 1329.92, 1365.91, 1365.91, 1383.95,
      1390.95, 1476.05, 1476.05, 1488.04, 1639.17, 1658.11, 1708.10, 1717.18, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36,
      1994.36, 2211.53, 2211.53, 2224.52, 2225.55, 2225.55, 2239.57, 2247.53, 2265.47, 2265.47, 2283.62, 2283.62, 2284.62,
      2297.63, 2299.62, 2319.42, 2384.42, 2705.69, 2807.88

      Matched peptides shown in Bold Red

           1 MGDCGCSGAS SCNCGSGCSC SNCGSK


2.    H41_ASHGO         Mass: 11331    Score: 11     Expect: 6.5e+002  Queries matched: 2
      Histone H4.1 - Ashbya gossypii (Yeast) (Eremothecium gossypii)
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      1320.85   1319.84   1319.72    0.12    46   - 56    1   ---  K.RISGLIYEDVR.A
      1708.10   1707.09   1707.02    0.08    22   - 36    1   ---  K.ILRDNIQGITKPAIR.R
      No match to: 807.56, 832.65, 842.67, 842.67, 854.21, 856.68, 856.68, 864.66, 865.18, 865.18, 870.71, 870.71, 877.21,
      887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1045.77, 1060.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76,
      1107.76, 1109.71, 1141.76, 1157.81, 1157.81, 1165.81, 1179.83, 1179.83, 1193.84, 1201.84, 1201.84, 1234.91, 1234.91,
      1265.88, 1277.95, 1277.95, 1300.78, 1302.96, 1307.93, 1307.93, 1323.92, 1329.92, 1365.91, 1365.91, 1383.95, 1390.95,
      1476.05, 1476.05, 1488.04, 1639.17, 1658.11, 1717.18, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 1994.36, 2211.53,
      2211.53, 2224.52, 2225.55, 2225.55, 2233.53, 2239.57, 2247.53, 2265.47, 2265.47, 2283.62, 2283.62, 2284.62, 2297.63,
      2299.62, 2319.42, 2384.42, 2705.69, 2807.88
```

```
Matched peptides shown in Bold Red

      1 MSGRGKGGKG LGKGGAKRHR KILRDNIQGI TKPAIRRLAR RGGVKRISGL
     51 IYEDVRAVLK SFLESVIRDA VTYTEHAKRK TVTSLDVVYA LKRQGRTLYG
    101 FGG
```

```
3.    COX18_KLULA        Mass: 34941     Score: 9       Expect: 9.5e+002  Queries matched: 4
      Inner membrane protein COX18, mitochondrial precursor (Cytochrome c oxidase assembly protein 18) -
     Observed  Mr(expt)  Mr(calc)    Delta Start    End Miss Ions Peptide
     1179.83   1178.82   1178.74     0.08     74  - 84    1   ---  R.KVVQSVPPVVK.L
     1179.83   1178.82   1178.74     0.08     74  - 84    1   ---  R.KVVQSVPPVVK.L
     1320.85   1319.84   1319.83     0.01     75  - 86    1   ---  K.VVQSVPPVVKLR.L
     2384.42   2383.42   2383.31     0.11    154  - 174   1   ---  K.NSVLPLVQVPLWVTMSMGLRK.L + Oxidation (M)
      No match to: 807.56, 832.65, 842.67, 842.67, 854.21, 856.68, 856.68, 864.66, 865.18, 865.18, 870.71, 870.71, 877.21,
      887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1045.77, 1060.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76,
      1107.76, 1109.71, 1141.76, 1157.81, 1157.81, 1165.81, 1193.84, 1201.84, 1201.84, 1234.91, 1234.91, 1265.88, 1277.95,
      1277.95, 1300.78, 1302.96, 1307.93, 1307.93, 1323.92, 1329.92, 1365.91, 1365.91, 1383.95, 1390.95, 1476.05, 1476.05,
      1488.04, 1639.17, 1658.11, 1708.10, 1717.18, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 1994.36, 2211.53, 2211.53,
      2224.52, 2225.55, 2225.55, 2233.53, 2239.57, 2247.53, 2265.47, 2265.47, 2283.62, 2283.62, 2284.62, 2297.63, 2299.62,
      2319.42, 2705.69, 2807.88
```

```
Matched peptides shown in Bold Red

      1 MLLRRLPAVH FAKGSRQFGS LQAIADSFVQ LHDASGVPWL VLIPTATFAL
     51 RTVFTLPLSI WQRKRIVKQQ ELRKVVQSVP PVVKLRLASM TAKANDEELT
    101 SSGSAIQTKE ETVGALQRGK RQLTPDQITM LSLKEMRKRQ KVLFKKYNVQ
    151 MWKNSVLPLV QVPLWVTMSM GLRKLTDSRL VDTNMPHAHV LQDLSETSWL
    201 THIGSLDLSL PLDAAPMLIP IILGTVSMIN VEYNGKTMQA TAVGTSGITT
    251 ATDTQSRTSQ TVNSILTATR LSTIFLIGVS TQASVLLSLY WITSQVYSLI
    301 QNRILDLLWP YQR
```

Mascot Search Results for C3-b

1. GRPE_NEUCR **Mass:** 26811 **Score:** 17 **Expect:** 1.5e+002 **Queries matched:** 5

GrpE protein homolog, mitochondrial precursor - Neurospora crassa

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1141.76	1140.75	1140.64	0.11	37	- 47	1	---	R.TAAPQLRSAAR.W
1165.80	1164.80	1164.73	0.06	226	- 236	0	---	R.VLRPAQVGVVK.N
1165.80	1164.80	1164.73	0.06	226	- 236	0	---	R.VLRPAQVGVVK.N
1235.76	1234.76	1234.68	0.07	167	- 177	0	---	K.MTESILLSTLK.K
2717.59	2716.59	2716.40	0.19	12	- 36	1	---	R.ALCSGARVAAQRPIASQIFQMQAAR.T + Carbamidomethyl (C); Oxidation (M)

No match to: 807.55, 832.64, 840.65, 842.66, 842.66, 847.61, 854.21, 855.21, 856.68, 856.68, 861.22, 864.17, 864.65, 865.18, 865.18, 868.68, 870.19, 870.70, 870.70, 871.19, 877.21, 887.16, 897.58, 919.12, 973.71, 973.71, 1033.71, 1033.71, 1045.76, 1045.76, 1057.75, 1060.77, 1060.77, 1065.71, 1066.71, 1090.73, 1106.74, 1107.75, 1109.71, 1121.79, 1140.78, 1157.81, 1157.81, 1158.80, 1179.82, 1179.82, 1184.83, 1194.81, 1201.84, 1232.83, 1234.91, 1234.91, 1265.87, 1265.87, 1300.77, 1307.92, 1307.92, 1320.83, 1323.91, 1357.95, 1365.89, 1365.89, 1381.90, 1383.94, 1390.94, 1476.05, 1476.05, 1498.04, 1658.10, 1708.09, 1717.17, 1717.17, 1792.06, 1792.06, 2211.52, 2211.52, 2225.53, 2265.45, 2384.40, 2384.40

Matched peptides shown in **Bold Red**

1 MLRTALTRSS R**ALCSGARVA AQRPIASQIF QMQAARTAAP QLRSAAR**WYS
51 AEAEGEKKAD EGAEQKEGET DEVAALKKQL EAKDAEAREW KDKCLRTVAD
101 FRNLQERTAR DVKQAKDFAI QKFAKDLVES VDNFERALSV VPQDKLKSEE
151 QSEHLKDLVN LYEGLK**MTES ILLSTLK**KHG LERIEPEGEV FNPNEHEATF
201 MAPMPDKEHN VVFHVQQKGF KLNGR**VLRPA QVGVVK**NK

2. RS21_ASPFU **Mass:** 10003 **Score:** 12 **Expect:** 5.4e+002 **Queries matched:** 3

40S ribosomal protein S21 - Aspergillus fumigatus (Sartorya fumigata)

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1708.09	1707.08	1706.97	0.11	23	- 38	1	---	R.IIKANDHASVQISIAK.V
1792.06	1791.06	1790.89	0.17	1	- 15	1	---	-.MENEKGEIVDLYVPR.K

```
1792.06   1791.06   1790.89    0.17    1   - 15   1   ---   -.MENEKGEIVDLYVPR.K

No match to: 807.55, 832.64, 840.65, 842.66, 842.66, 847.61, 854.21, 855.21, 856.68, 856.68, 861.22, 864.17, 864.65,
865.18, 865.18, 868.68, 870.19, 870.70, 870.70, 871.19, 877.21, 887.16, 897.58, 919.12, 973.71, 973.71, 1033.71, 1033.71,
1045.76, 1045.76, 1057.75, 1060.77, 1060.77, 1065.71, 1066.71, 1090.73, 1106.74, 1107.75, 1109.71, 1121.79, 1140.78,
1141.76, 1157.81, 1157.81, 1158.80, 1165.80, 1165.80, 1179.82, 1179.82, 1184.83, 1194.81, 1201.84, 1232.83, 1234.91,
1234.91, 1235.76, 1265.87, 1265.87, 1300.77, 1307.92, 1307.92, 1320.83, 1323.91, 1357.95, 1365.89, 1365.89, 1381.90,
1383.94, 1390.94, 1476.05, 1476.05, 1498.04, 1658.10, 1717.17, 1717.17, 2211.52, 2211.52, 2225.53, 2265.45, 2384.40,
2384.40, 2717.59


 Matched peptides shown in Bold Red

     1 MENEKGEIVD LYVPRKCSAT NRIIKANDHA SVQISIAKVD ENGRYTGENQ
    51 SYALCGFIRA RGESDDSLNR LCQRDGYIRN VWSASRQR


3.    H41_ASHGO         Mass: 11331    Score: 11     Expect: 6.3e+002  Queries matched: 2
      Histone H4.1 - Ashbya gossypii (Yeast) (Eremothecium gossypii)
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      1320.83   1319.83   1319.72    0.11   46   - 56   1   ---   K.RISGLIYEDVR.A
      1708.09   1707.08   1707.02    0.07   22   - 36   1   ---   K.ILRDNIQGITKPAIR.R
      No match to: 807.55, 832.64, 840.65, 842.66, 842.66, 847.61, 854.21, 855.21, 856.68, 856.68, 861.22, 864.17, 864.65,
      865.18, 865.18, 868.68, 870.19, 870.70, 870.70, 871.19, 877.21, 887.16, 897.58, 919.12, 973.71, 973.71, 1033.71, 1033.71,
      1045.76, 1045.76, 1057.75, 1060.77, 1060.77, 1065.71, 1066.71, 1090.73, 1106.74, 1107.75, 1109.71, 1121.79, 1140.78,
      1141.76, 1157.81, 1157.81, 1158.80, 1165.80, 1165.80, 1179.82, 1179.82, 1184.83, 1194.81, 1201.84, 1232.83, 1234.91,
      1234.91, 1235.76, 1265.87, 1265.87, 1300.77, 1307.92, 1307.92, 1323.91, 1357.95, 1365.89, 1365.89, 1381.90, 1383.94,
      1390.94, 1476.05, 1476.05, 1498.04, 1658.10, 1717.17, 1717.17, 1792.06, 1792.06, 2211.52, 2211.52, 2225.53, 2265.45,
      2384.40, 2384.40, 2717.59


       Matched peptides shown in Bold Red

           1 MSGRGKGGKG LGKGGAKRHR KILRDNIQGI TKPAIRRLAR RGGVKRISGL
          51 IYEDVRAVLK SFLESVIRDA VTYTEHAKRK TVTSLDVVYA LKRQGRTLYG
         101 FGG
```

Mascot Search Results for C3-c

```
1.    MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
      Metallothionein (MT) - Neurospora crassa
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      2233.50   2232.50   2232.67    -0.18    2   - 26    0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
      No match to: 807.55, 840.64, 842.66, 842.66, 854.21, 856.67, 856.67, 858.66, 864.65, 865.17, 865.17, 868.68, 870.70,
      870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79,
      1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1193.83, 1201.83, 1201.83, 1234.90, 1234.90, 1265.86, 1277.94, 1277.94,
      1300.77, 1302.94, 1307.91, 1307.91, 1329.89, 1365.89, 1365.89, 1383.93, 1383.93, 1390.93, 1435.03, 1476.03, 1476.03,
      1488.02, 1639.15, 1658.10, 1708.09, 1717.16, 1766.08, 1795.13, 1795.13, 1839.27, 1941.29, 1994.34, 2210.51, 2211.51,
      2211.51, 2223.50, 2225.53, 2225.53, 2247.51, 2265.45, 2265.45, 2279.47, 2283.60, 2283.60, 2284.60, 2297.61, 2297.61,
      2298.61, 2299.60, 2306.57, 2338.54, 2384.42


       Matched peptides shown in Bold Red

            1 MGDCGCSGAS SCNCGSGCSC SNCGSK


2.    RS9_PODAN         Mass: 21762    Score: 11     Expect: 6e+002  Queries matched: 5
      40S ribosomal protein S9 (S7) - Podospora anserina
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      1201.83   1200.83   1200.72    0.11  119    - 128   1   ---  K.SIHHARVLIR.Q
      1201.83   1200.83   1200.72    0.11  119    - 128   1   ---  K.SIHHARVLIR.Q
      1300.77   1299.77   1299.69    0.08   56    - 66    1   ---  R.ELLTLDEKDPK.R
      1795.13   1794.13   1793.96    0.17   91    - 105   1   ---  K.LDYVLALKAEDFLER.R
      1795.13   1794.13   1793.96    0.17   91    - 105   1   ---  K.LDYVLALKAEDFLER.R
      No match to: 807.55, 840.64, 842.66, 842.66, 854.21, 856.67, 856.67, 858.66, 864.65, 865.17, 865.17, 868.68, 870.70,
      870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79,
      1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1193.83, 1234.90, 1234.90, 1265.86, 1277.94, 1277.94, 1302.94, 1307.91,
      1307.91, 1329.89, 1365.89, 1365.89, 1383.93, 1383.93, 1390.93, 1435.03, 1476.03, 1476.03, 1488.02, 1639.15, 1658.10,
      1708.09, 1717.16, 1766.08, 1839.27, 1941.29, 1994.34, 2210.51, 2211.51, 2211.51, 2223.50, 2225.53, 2225.53, 2233.50,
      2247.51, 2265.45, 2265.45, 2279.47, 2283.60, 2283.60, 2284.60, 2297.61, 2297.61, 2298.61, 2299.60, 2306.57, 2338.54,
```

2384.42

Matched peptides shown in **Bold Red**

1 MAPRSYSKTA KVPRRPFEAA RLDSELKLVG EYGLRNKREV WRVLLTLSKI
51 RRAAR**ELLTL DEKDPK**RLFE GNALIRRLVR VGVLDESRMK **LDYVLALKAE**
101 **DFLER**RLQTL VYKLGLAK**SI HHARVLIR**QR HIRVGKQIVN VPSFVVRLDS
151 QKHIDFALTS PFGGGRPGRV RRKKAKAAEG GDGDAEEDEE

3. FIS1_KLULA **Mass:** 17527 **Score:** 9 **Expect:** 8.9e+002 **Queries matched:** 2
Mitochondria fission 1 protein - Kluyveromyces lactis (Yeast) (Candida sphaerica)

```
Observed   Mr(expt)   Mr(calc)    Delta Start   End Miss Ions Peptide
1766.08    1765.08    1764.93     0.14    56   - 70    1   ---  K.EDQMLGVKLLTDIYK.E
1994.34    1993.33    1993.23     0.10   131   - 151   0   ---  K.GIALISAGIAIGATTIGLLIR.G
```

No match to: 807.55, 840.64, 842.66, 842.66, 854.21, 856.67, 856.67, 858.66, 864.65, 865.17, 865.17, 868.68, 870.70, 870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79, 1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1193.83, 1201.83, 1201.83, 1234.90, 1234.90, 1265.86, 1277.94, 1277.94, 1300.77, 1302.94, 1307.91, 1307.91, 1329.89, 1365.89, 1365.89, 1383.93, 1383.93, 1390.93, 1435.03, 1476.03, 1476.03, 1488.02, 1639.15, 1658.10, 1708.09, 1717.16, 1795.13, 1795.13, 1839.27, 1941.29, 2210.51, 2211.51, 2211.51, 2223.50, 2225.53, 2225.53, 2233.50, 2247.51, 2265.45, 2265.45, 2279.47, 2283.60, 2283.60, 2284.60, 2297.61, 2297.61, 2298.61, 2299.60, 2306.57, 2338.54, 2384.42

Matched peptides shown in **Bold Red**

1 MTNIHYLPEL EDSYSALNSD QIEILRQQVL NEGGEISSIQ SRFNYAWGLV
51 RSTNK**EDQML GVKLLTDIYK** ESPMRRRECL YYLTIGCYKL GEYSTAKRYV
101 DALVHHEPEN KQALMLQTAV ENKITSQGLK **GIALISAGIA IGATTIGLLI**
151 **R**GRRR

{MATRIX SCIENCE} Mascot Search Results for C4-a

```
1.    MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
      Metallothionein (MT) - Neurospora crassa
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      2233.49   2232.48   2232.67   -0.19    2   - 26    0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
      No match to: 807.55, 807.55, 817.63, 832.64, 842.66, 842.66, 847.60, 856.67, 856.67, 864.65, 865.17, 868.68, 870.69,
      870.69, 877.20, 880.51, 887.16, 973.71, 973.71, 1033.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1065.71, 1090.73,
      1106.75, 1107.74, 1109.69, 1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1184.82, 1193.83, 1201.83, 1201.83, 1234.90,
      1234.90, 1262.83, 1265.86, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1329.89, 1365.89, 1365.89, 1381.90, 1383.93,
      1390.93, 1435.02, 1476.03, 1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1708.08, 1717.15, 1717.15, 1839.26, 1994.33,
      2194.57, 2211.51, 2211.51, 2223.53, 2225.52, 2225.52, 2239.54, 2247.50, 2265.45, 2265.45, 2283.59, 2283.59, 2284.59,
      2297.60, 2299.60, 2305.59, 2367.68, 2384.39


       Matched peptides shown in Bold Red

           1 MGDCGCSGAS SCNCGSGCSC SNCGSK


2.    DAD3_KLULA        Mass: 9810     Score: 11     Expect: 5.6e+002  Queries matched: 3
      DASH complex subunit DAD3 (Outer kinetochore protein DAD3) - Kluyveromyces lactis (Yeast) (Candida
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      880.51    879.50    879.48     0.02   11    - 17    1   ---  R.SVLDKYR.F
      973.71    972.70    972.62     0.08   56    - 64    0   ---  K.ISLVSTLLK.G
      973.71    972.70    972.62     0.08   56    - 64    0   ---  K.ISLVSTLLK.G
      No match to: 807.55, 807.55, 817.63, 832.64, 842.66, 842.66, 847.60, 856.67, 856.67, 864.65, 865.17, 868.68, 870.69,
      870.69, 877.20, 887.16, 1033.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1065.71, 1090.73, 1106.75, 1107.74,
      1109.69, 1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1184.82, 1193.83, 1201.83, 1201.83, 1234.90, 1234.90, 1262.83,
      1265.86, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1329.89, 1365.89, 1365.89, 1381.90, 1383.93, 1390.93, 1435.02,
      1476.03, 1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1708.08, 1717.15, 1717.15, 1839.26, 1994.33, 2194.57, 2211.51,
      2211.51, 2223.53, 2225.52, 2225.52, 2233.49, 2239.54, 2247.50, 2265.45, 2265.45, 2283.59, 2283.59, 2284.59, 2297.60,
      2299.60, 2305.59, 2367.68, 2384.39
```

```
Matched peptides shown in Bold Red

     1 MSSELTPLQR SVLDKYRFLA ESLRELSETL SDLNNTHEDS KPEAILQEMR
    51 EIEVKISLVS TLLKGSVYSL VIQRRMDHQQ KPSDL
```

```
3.     H2A_USTMA          Mass: 14191    Score: 9      Expect: 1e+003  Queries matched: 3
       Histone H2A - Ustilago maydis (Smut fungus)
       Observed  Mr(expt)  Mr(calc)   Delta  Start   End  Miss  Ions  Peptide
       807.55    806.54    806.52     0.02   34    - 39    1    ---   R.IHRLLR.K
       807.55    806.54    806.52     0.02   34    - 39    1    ---   R.IHRLLR.K
       1329.89   1328.88   1328.84    0.04   82    - 92    1    ---   R.IIPRHLQLAIR.N
       No match to: 817.63, 832.64, 842.66, 842.66, 847.60, 856.67, 856.67, 864.65, 865.17, 868.68, 870.69, 870.69, 877.20,
       880.51, 887.16, 973.71, 973.71, 1033.71, 1033.71, 1037.72, 1045.76, 1045.76, 1060.76, 1065.71, 1090.73, 1106.75, 1107.74,
       1109.69, 1157.80, 1165.80, 1165.80, 1179.81, 1179.81, 1184.82, 1193.83, 1201.83, 1201.83, 1234.90, 1234.90, 1262.83,
       1265.86, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1365.89, 1365.89, 1381.90, 1383.93, 1390.93, 1435.02, 1476.03,
       1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1708.08, 1717.15, 1717.15, 1839.26, 1994.33, 2194.57, 2211.51, 2211.51,
       2223.53, 2225.52, 2225.52, 2233.49, 2239.54, 2247.50, 2265.45, 2265.45, 2283.59, 2283.59, 2284.59, 2297.60, 2299.60,
       2305.59, 2367.68, 2384.39
```

```
Matched peptides shown in Bold Red

     1 MSSGGKSGGK AGDASSKAQS RSAKAGLQFP VGRIHRLLRK GNYAQRVGAG
    51 APVYLAAVLE YLAAEILELA GNAARDNKKS RIIPRHLQLA IRNDEELNKL
   101 LGGVTISQGG VLPFIQSELL PAKSGKPKKA GGSQDI
```

Mascot Search Results for C4-b

```
1.     H41_ASHGO         Mass: 11331    Score: 13     Expect: 3.6e+002  Queries matched: 3
       Histone H4.1 - Ashbya gossypii (Yeast) (Eremothecium gossypii)
       Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
       1320.83   1319.83   1319.72    0.11   46   - 56   1   ---  K.RISGLIYEDVR.A
       1708.08   1707.07   1707.02    0.05   22   - 36   1    3   K.ILRDNIQGITKPAIR.R
       1708.08   1707.07   1707.02    0.05   22   - 36   1   ---  K.ILRDNIQGITKPAIR.R
       No match to: 807.55, 825.26, 832.64, 842.66, 842.66, 845.25, 847.60, 855.21, 856.68, 856.68, 861.23, 864.65, 865.17,
       865.17, 868.68, 870.70, 870.70, 877.20, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1045.76, 1057.76, 1060.76,
       1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1157.80, 1165.80, 1179.81, 1179.81, 1193.83,
       1201.83, 1232.82, 1234.91, 1234.91, 1265.86, 1277.94, 1307.91, 1307.91, 1323.91, 1365.89, 1365.89, 1383.93, 1435.03,
       1476.03, 1476.03, 1488.02, 1639.16, 1658.10, 1717.16, 1792.05, 1792.05, 1839.27, 1941.28, 2194.55, 2211.51, 2211.51,
       2223.53, 2225.53, 2225.53, 2233.49, 2239.54, 2247.52, 2255.48, 2265.45, 2265.45, 2283.60, 2283.60, 2284.59, 2298.60,
       2384.40, 2384.40, 2705.68


      Matched peptides shown in Bold Red

           1 MSGRGKGGKG LGKGGAKRHR KILRDNIQGI TKPAIRRLAR RGGVKRISGL
          51 IYEDVRAVLK SFLESVIRDA VTYTEHAKRK TVTSLDVVYA LKRQGRTLYG
         101 FGG


2.     MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
       Metallothionein (MT) - Neurospora crassa
       Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
       2233.49   2232.49   2232.67   -0.19    2   - 26   0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
       No match to: 807.55, 825.26, 832.64, 842.66, 842.66, 845.25, 847.60, 855.21, 856.68, 856.68, 861.23, 864.65, 865.17,
       865.17, 868.68, 870.70, 870.70, 877.20, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1045.76, 1057.76, 1060.76,
       1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1157.80, 1165.80, 1179.81, 1179.81, 1193.83,
       1201.83, 1232.82, 1234.91, 1234.91, 1265.86, 1277.94, 1307.91, 1307.91, 1320.83, 1323.91, 1365.89, 1365.89, 1383.93,
       1435.03, 1476.03, 1476.03, 1488.02, 1639.16, 1658.10, 1708.08, 1708.08, 1717.16, 1792.05, 1792.05, 1839.27, 1941.28,
       2194.55, 2211.51, 2211.51, 2223.53, 2225.53, 2225.53, 2239.54, 2247.52, 2255.48, 2265.45, 2265.45, 2283.60, 2283.60,
```

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2284.59, 2298.60, 2384.40, 2384.40, 2705.68


 Matched peptides shown in Bold Red

      1 MGDCGCSGAS SCNCGSGCSC SNCGSK



3.    RS21_ASPFU        Mass: 10003    Score: 11     Expect: 5.6e+002  Queries matched: 4
      40S ribosomal protein S21 - Aspergillus fumigatus (Sartorya fumigata)
     Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
     1708.08   1707.07   1706.97    0.10   23   - 38   1   ---  R.IIKANDHASVQISIAK.V
     1708.08   1707.07   1706.97    0.10   23   - 38   1   ---  R.IIKANDHASVQISIAK.V
     1792.05   1791.05   1790.89    0.16    1   - 15   1   ---  -.MENEKGEIVDLYVPR.K
     1792.05   1791.05   1790.89    0.16    1   - 15   1   ---  -.MENEKGEIVDLYVPR.K
      No match to: 807.55, 825.26, 832.64, 842.66, 842.66, 845.25, 847.60, 855.21, 856.68, 856.68, 861.23, 864.65, 865.17,
      865.17, 868.68, 870.70, 870.70, 877.20, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1045.76, 1057.76, 1060.76,
      1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1157.80, 1165.80, 1179.81, 1179.81, 1193.83,
      1201.83, 1232.82, 1234.91, 1234.91, 1265.86, 1277.94, 1307.91, 1307.91, 1320.83, 1323.91, 1365.89, 1365.89, 1383.93,
      1435.03, 1476.03, 1476.03, 1488.02, 1639.16, 1658.10, 1717.16, 1839.27, 1941.28, 2194.55, 2211.51, 2211.51, 2223.53,
      2225.53, 2225.53, 2233.49, 2239.54, 2247.52, 2255.48, 2265.45, 2265.45, 2283.60, 2283.60, 2284.59, 2298.60, 2384.40,
      2384.40, 2705.68


 Matched peptides shown in Bold Red

      1 MENEKGEIVD LYVPRKCSAT NRIIKANDHA SVQISIAKVD ENGRYTGENQ
     51 SYALCGFIRA RGESDDSLNR LCQRDGYIRN VWSASRQR
```

Mascot Search Results for C4-c

```
1.     MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
       Metallothionein (MT) - Neurospora crassa
      Observed   Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      2233.48    2232.47   2232.67    -0.20    2  - 26    0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
       No match to: 807.55, 825.26, 835.21, 842.66, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 865.18,
       868.68, 870.18, 870.69, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 920.10, 930.58, 930.58, 973.71, 1023.71,
       1023.71, 1033.70, 1037.72, 1045.75, 1045.75, 1057.75, 1060.76, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76,
       1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1179.81, 1201.84, 1234.90, 1234.90, 1265.85, 1265.85, 1300.77,
       1307.91, 1307.91, 1323.90, 1365.88, 1365.88, 1383.93, 1435.03, 1476.03, 1476.03, 1658.08, 1708.08, 1717.14, 1717.14,
       1792.04, 1795.12, 2211.49, 2211.49, 2225.51, 2225.51, 2239.50, 2248.51, 2265.42, 2265.42, 2279.45, 2283.56, 2283.56,
       2299.58, 2319.36, 2384.37, 2384.37


        Matched peptides shown in Bold Red

            1 ERHVGRKSTN NLNLTCQWGT CNTTTVKRDH ITSHIRVHVP LKPHKCD


2.     RS9_PODAN         Mass: 21762    Score: 12     Expect: 5.1e+002  Queries matched: 3
       40S ribosomal protein S9 (S7) - Podospora anserina
      Observed   Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      1201.84    1200.83   1200.72    0.11   119  - 128   1   ---  K.SIHHARVLIR.Q
      1300.77    1299.77   1299.69    0.07    56  - 66    1   ---  R.ELLTLDEKDPK.R
      1795.12    1794.11   1793.96    0.15    91  - 105   1   ---  K.LDYVLALKAEDFLER.R
       No match to: 807.55, 825.26, 835.21, 842.66, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 865.18,
       868.68, 870.18, 870.69, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 920.10, 930.58, 930.58, 973.71, 1023.71,
       1023.71, 1033.70, 1037.72, 1045.75, 1045.75, 1057.75, 1060.76, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76,
       1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1179.81, 1234.90, 1234.90, 1265.85, 1265.85, 1307.91, 1307.91,
       1323.90, 1365.88, 1365.88, 1383.93, 1435.03, 1476.03, 1476.03, 1658.08, 1708.08, 1717.14, 1717.14, 1792.04, 2211.49,
       2211.49, 2225.51, 2225.51, 2233.48, 2239.50, 2248.51, 2265.42, 2265.42, 2279.45, 2283.56, 2283.56, 2299.58, 2319.36,
       2384.37, 2384.37
```

Matched peptides shown in **Bold Red**

1 MAPRSYSKTA KVPRRPFEAA RLDSELKLVG EYGLRNKREV WRVLLTLSKI
51 RRAAR**ELLTL DEKDPK**RLFE GNALIRRLVR VGVLDESRMK **LDYVLALKAE**
101 **DFLER**RLQTL VYKLGLAK**SI HHARVLIR**QR HIRVGKQIVN VPSFVVRLDS
151 QKHIDFALTS PFGGGRPGRV RRKKAKAAEG GDGDAEEDEE

3. RS21_ASPFU **Mass:** 10003 **Score:** 11 **Expect:** 5.6e+002 **Queries matched:** 2
40S ribosomal protein S21 - Aspergillus fumigatus (Sartorya fumigata)

```
Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
1708.08   1707.07   1706.97    0.10   23    - 38    1   ---  R.IIKANDHASVQISIAK.V
1792.04   1791.03   1790.89    0.14    1    - 15    1   ---  -.MENEKGEIVDLYVPR.K
```

No match to: 807.55, 825.26, 835.21, 842.66, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 865.18, 868.68, 870.18, 870.69, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 920.10, 930.58, 930.58, 973.71, 1023.71, 1023.71, 1033.70, 1037.72, 1045.75, 1045.75, 1057.75, 1060.76, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76, 1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1179.81, 1201.84, 1234.90, 1234.90, 1265.85, 1265.85, 1300.77, 1307.91, 1307.91, 1323.90, 1365.88, 1365.88, 1383.93, 1435.03, 1476.03, 1476.03, 1658.08, 1717.14, 1717.14, 1795.12, 2211.49, 2211.49, 2225.51, 2225.51, 2233.48, 2239.50, 2248.51, 2265.42, 2265.42, 2279.45, 2283.56, 2283.56, 2299.58, 2319.36, 2384.37, 2384.37

Matched peptides shown in **Bold Red**

1 **MENEKGEIVD LYVPR**KCSAT NR**IIKANDHA SVQISIAK**VD ENGRYTGENQ
51 SYALCGFIRA RGESDDSLNR LCQRDGYIRN VWSASRQR

Mascot Search Results for C5-a

```
1.    MT_NEUCR          Mass: 2364     Score: 12     Expect: 4.8e+002  Queries matched: 1
      Metallothionein (MT) - Neurospora crassa
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      2233.46   2232.45   2232.67   -0.22    2   - 26    0   ---  M.GDCGCSGASSCNCGSGCSCSNCGSK.-
      No match to: 807.54, 825.24, 832.63, 835.19, 842.65, 842.65, 847.59, 854.19, 855.19, 856.66, 856.66, 861.21, 864.15,
      864.64, 865.16, 865.16, 868.67, 870.68, 870.68, 877.19, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69,
      1033.69, 1036.71, 1037.71, 1045.74, 1045.74, 1057.75, 1060.74, 1060.74, 1065.69, 1067.72, 1090.71, 1106.72, 1107.72,
      1109.68, 1121.77, 1157.78, 1157.78, 1165.78, 1165.78, 1179.79, 1179.79, 1193.81, 1201.81, 1201.81, 1234.89, 1234.89,
      1265.84, 1265.84, 1300.76, 1307.89, 1307.89, 1320.81, 1323.89, 1365.87, 1365.87, 1381.88, 1383.91, 1391.91, 1435.00,
      1476.01, 1476.01, 1498.02, 1658.07, 1708.05, 1717.13, 1792.03, 2211.47, 2211.47, 2225.49, 2225.49, 2239.50, 2248.51,
      2265.40, 2284.54, 2384.35, 2384.35


       Matched peptides shown in Bold Red

            1 MGDCGCSGAS SCNCGSGCSC SNCGSK


2.    MBF1_KLULA        Mass: 16436    Score: 12     Expect: 5.1e+002  Queries matched: 4
      Multiprotein-bridging factor 1 - Kluyveromyces lactis (Yeast) (Candida sphaerica)
      Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
      1067.72   1066.72   1066.61    0.10   118  - 127   0   ---  R.GIPNQQILGK.M
      1109.68   1108.68   1108.62    0.05   140  - 150   1   ---  K.NIGEPLGGPKK.-
      1157.78   1156.77   1156.68    0.09    77  - 87    1   ---  K.IDVSVGKAIQK.G
      1157.78   1156.77   1156.68    0.09    77  - 87    1   ---  K.IDVSVGKAIQK.G
      No match to: 807.54, 825.24, 832.63, 835.19, 842.65, 842.65, 847.59, 854.19, 855.19, 856.66, 856.66, 861.21, 864.15,
      864.64, 865.16, 865.16, 868.67, 870.68, 870.68, 877.19, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69,
      1033.69, 1036.71, 1037.71, 1045.74, 1045.74, 1057.75, 1060.74, 1060.74, 1065.69, 1090.71, 1106.72, 1107.72, 1121.77,
      1165.78, 1165.78, 1179.79, 1179.79, 1193.81, 1201.81, 1201.81, 1234.89, 1234.89, 1265.84, 1265.84, 1300.76, 1307.89,
      1307.89, 1320.81, 1323.89, 1365.87, 1365.87, 1381.88, 1383.91, 1391.91, 1435.00, 1476.01, 1476.01, 1498.02, 1658.07,
      1708.05, 1717.13, 1792.03, 2211.47, 2211.47, 2225.49, 2225.49, 2233.46, 2239.50, 2248.51, 2265.40, 2284.54, 2384.35,
      2384.35
```

Matched peptides shown in **Bold Red**

1 MSEWEPSTVI GRKVRIGGGG PRQQVARTQG QINEARRSGM VLSVDKKYGT
51 TNSKSSPEGQ RLTKVDRETD IVKPKK**IDVS VGKAIQK**GRQ DKNLTQKDLA
101 TKINEKPTIV NDYESGR**GIP NQQILGK**MER ALGIKLRGK**N IGEPLGGPKK**

3. H2B_ROSNE **Mass:** 14789 **Score:** 12 **Expect:** 5.3e+002 **Queries matched:** 4

Histone H2B - Rosellinia necatrix (White root-rot fungus)

```
Observed  Mr(expt)  Mr(calc)   Delta  Start   End  Miss  Ions  Peptide
807.54    806.53    806.46     0.07    90   - 96    1    ---   K.LAAYNKK.S
1067.72   1066.72   1066.65    0.07     9   - 19    1    ---   K.KPAAKAPVASK.A
1365.87   1364.86   1364.73    0.13    83   - 95    1    ---   R.VATEASKLAAYNK.K
1365.87   1364.86   1364.73    0.13    83   - 95    1    ---   R.VATEASKLAAYNK.K
```

No match to: 825.24, 832.63, 835.19, 842.65, 842.65, 847.59, 854.19, 855.19, 856.66, 856.66, 861.21, 864.15, 864.64, 865.16, 865.16, 868.67, 870.68, 870.68, 877.19, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69, 1033.69, 1036.71, 1037.71, 1045.74, 1045.74, 1057.75, 1060.74, 1060.74, 1065.69, 1090.71, 1106.72, 1107.72, 1109.68, 1121.77, 1157.78, 1157.78, 1165.78, 1165.78, 1179.79, 1179.79, 1193.81, 1201.81, 1201.81, 1234.89, 1234.89, 1265.84, 1265.84, 1300.76, 1307.89, 1307.89, 1320.81, 1323.89, 1381.88, 1383.91, 1391.91, 1435.00, 1476.01, 1476.01, 1498.02, 1658.07, 1708.05, 1717.13, 1792.03, 2211.47, 2211.47, 2225.49, 2225.49, 2233.46, 2239.50, 2248.51, 2265.40, 2284.54, 2384.35, 2384.35

Matched peptides shown in **Bold Red**

1 MPPKAADK**KP AAKAPVASK**A PEKKDAGKKT ASTGEKKKRT KARRETYSSY
51 IYKVLKQVHP DTGISNRAMS ILNSFVNDIF ER**VATEASKL AAYNKK**STIS
101 SREIQTSVRL ILPGELAKHA VSEGTKAVTK YSSSTK

Mascot Search Results for C5-b

```
1.     PGK_KLULA         Mass: 44487    Score: 19     Expect: 1.1e+002  Queries matched: 7
       Phosphoglycerate kinase (EC 2.7.2.3) - Kluyveromyces lactis (Yeast) (Candida sphaerica)
       Observed  Mr(expt)  Mr(calc)   Delta Start    End Miss Ions Peptide
       1106.71   1105.71   1105.61    0.09   320  - 330  1    ---  R.KAFAATVAEAK.T
       1109.68   1108.67   1108.62    0.06     1  - 10   1    ---  -.MSLSSKLTVK.D + Oxidation (M)
       1184.79   1183.78   1183.68    0.10   219  - 228  0    ---  K.IQLIDNLLDK.V
       1316.74   1315.73   1315.74   -0.00   278  - 289  0    ---  K.IVLPTDFVIGDK.F
       1320.80   1319.79   1319.67    0.12    75  - 86   0    ---  K.YSLAPVADELSR.L
       1320.80   1319.79   1319.67    0.12    75  - 86   0    ---  K.YSLAPVADELSR.L
       1332.79   1331.78   1331.71    0.07   405  - 416  1    ---  K.ELPGVTFLSNKQ.-
       No match to: 807.53, 825.23, 832.62, 835.19, 842.64, 842.64, 847.58, 854.19, 855.19, 856.66, 856.66, 861.20, 864.63,
       865.15, 865.15, 868.66, 870.16, 870.68, 870.68, 871.17, 877.18, 887.14, 887.14, 903.12, 913.55, 973.69, 993.66, 1033.68,
       1033.68, 1037.69, 1045.73, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1107.72, 1107.72, 1157.77,
       1165.77, 1165.77, 1179.79, 1179.79, 1193.81, 1193.81, 1201.81, 1201.81, 1232.80, 1234.88, 1234.88, 1235.73, 1254.81,
       1265.83, 1265.83, 1307.88, 1323.88, 1329.85, 1365.86, 1365.86, 1381.86, 1390.89, 1434.99, 1476.01, 1476.01, 1658.05,
       1708.04, 1708.04, 1717.12, 1742.00, 1792.02, 1792.02, 1795.09, 2211.46, 2211.46, 2225.47, 2384.33


       Matched peptides shown in Bold Red

            1 MSLSSKLTVK DLDVTGKRVF IRVDFNVPLD GKKITSNQRI VAALPTIQYV
           51 LEKKPKAIVL ASHLGRPNGE VNDKYSLAPV ADELSRLLQK PVTFLHDCVG
          101 EEVTNAVNNA KDGEVFLLEN LRFHIEEEGS RKVDGNKVKA DKAAVTKFRE
          151 QLSSLADVYV NDAFGTAHRA HSSIVGFDLP NRAAGFLLSK ELQYFAKALE
          201 NPTRPFLAIL GGAKVADKIQ LIDNLLDKVD SLIIGGGMAF TFKKVLENTE
          251 IGDSIYDAAG AELVPKLVEK AKKNNVKIVL PTDFVIGDKF SADANTKVVT
          301 DKEGIPSGWQ GLDNGPESRK AFAATVAEAK TIVWNGPPGV FEFAPFAKGT
          351 EALLDAVVAS SQAGNTVIIG GGDTATVAKK YGVVDKISHV STGGGASLEL
          401 LEGKELPGVT FLSNKQ
```

```
2.     RFC5_NEUCR         Mass: 39895    Score: 18     Expect: 1.1e+002  Queries matched: 9
       Replication factor C subunit 5 (Replication factor C5) (Probable activator 1 subunit 5) - Neurospor
       Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
       913.55    912.55    912.56    -0.02   106  - 113  0   ---  R.VVVQDLLK.E
       1232.80   1231.79   1231.68    0.11    58  - 68   1   ---  K.ELYGPGVEKIK.I
       1307.88   1306.87   1306.75    0.12   179  - 190  1   ---  R.CLLVRVAAPTHK.E
       1320.80   1319.79   1319.68    0.11   191  - 202  1   ---  K.EICDVLASSAKK.E + Carbamidomethyl (C)
       1320.80   1319.79   1319.68    0.11   191  - 202  1   ---  K.EICDVLASSAKK.E + Carbamidomethyl (C)
       1365.86   1364.85   1364.74    0.11   131  - 142  0   ---  K.VVVINEADHLTR.D
       1365.86   1364.85   1364.74    0.11   131  - 142  0   ---  K.VVVINEADHLTR.D
       1708.04   1707.03   1706.93    0.10   328  - 341  1   ---  K.VIFHLEAFVAKFMR.I
       1708.04   1707.03   1706.93    0.10   328  - 341  1   ---  K.VIFHLEAFVAKFMR.I
       No match to: 807.53, 825.23, 832.62, 835.19, 842.64, 842.64, 847.58, 854.19, 855.19, 856.66, 856.66, 861.20, 864.63,
       865.15, 865.15, 868.66, 870.16, 870.68, 870.68, 871.17, 877.18, 887.14, 887.14, 903.12, 973.69, 993.66, 1033.68, 1033.68,
       1037.69, 1045.73, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1106.71, 1107.72, 1107.72, 1109.68,
       1157.77, 1165.77, 1165.77, 1179.79, 1179.79, 1184.79, 1193.81, 1193.81, 1201.81, 1201.81, 1234.88, 1234.88, 1235.73,
       1254.81, 1265.83, 1265.83, 1316.74, 1323.88, 1329.85, 1332.79, 1381.86, 1390.89, 1434.99, 1476.01, 1476.01, 1658.05,
       1717.12, 1742.00, 1792.02, 1792.02, 1795.09, 2211.46, 2211.46, 2225.47, 2384.33
```

Matched peptides shown in **Bold Red**

```
      1 MALIVDKHRP RSLDALTYHT ELSERLRSLA QSGDFPHLLV YGPSGAGKKT
     51 RIVATLKELY GPGVEKIKID ARVFQTSSNR KLEFNIVASV YHLEITPSDV
    101 GNYDRVVVQD LLKEVAQTQQ VDLSAKQRFK VVVINEADHL TRDAQAALRR
    151 TMEKYSPNLR LILLANSTAN IIAPIRSRCL LVRVAAPTHK EICDVLASSA
    201 KKEGWPIVKG LHQRIAEESG RNLRRALLMY EAVYAQNEKV TDSTPIPPPD
    251 WEALIGQIAK EIMEEHTPAR ILQVRAKLYD LLTHCIPATI ILKTLTFKLI
    301 PLIDDALKAD VIYWSAFYEH RIRTGTKVIF HLEAFVAKFM RIFEMYLMSM
    351 DL
```

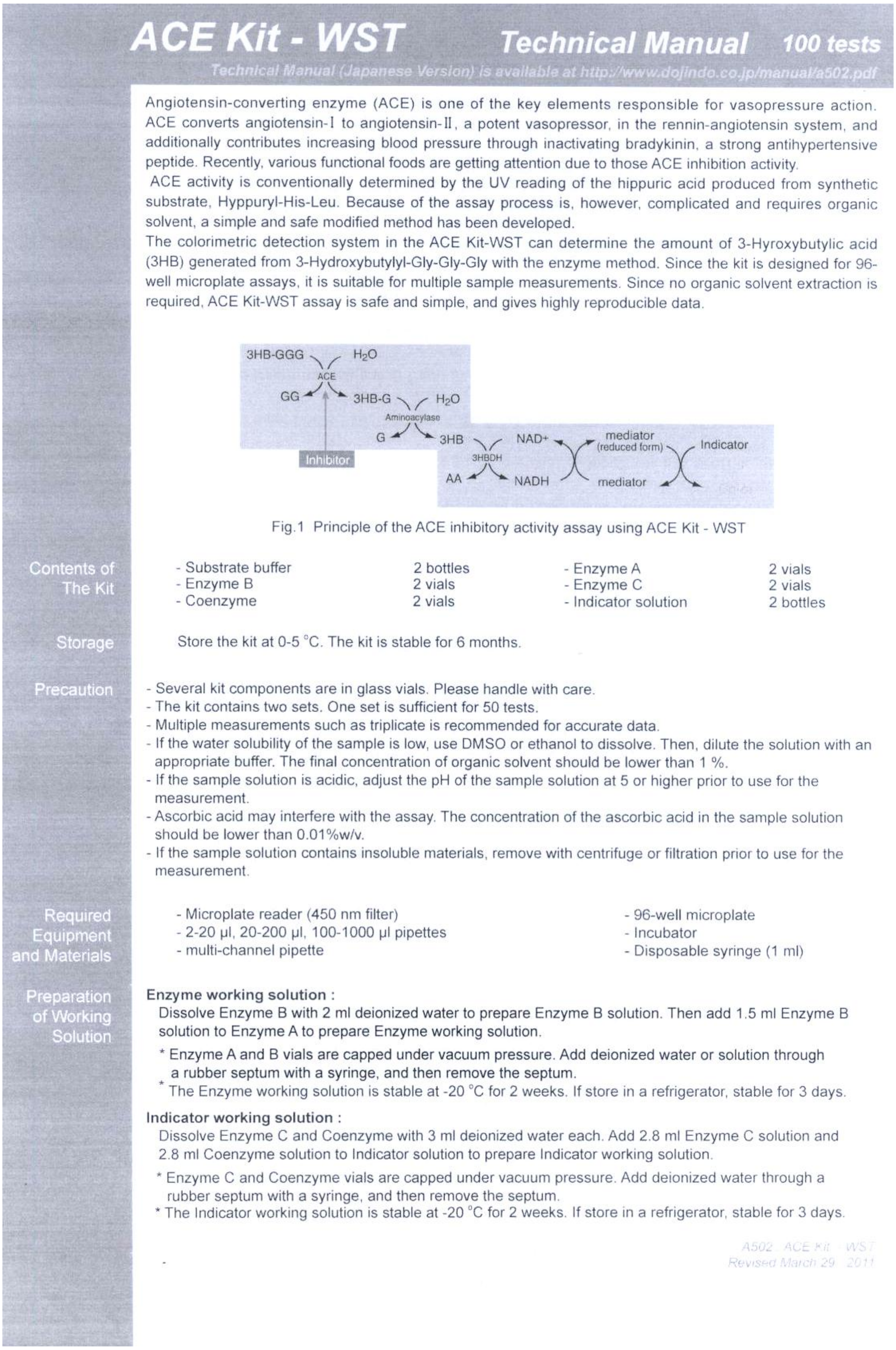
```
3.      RMAR_WILMR        Mass: 44348    Score: 14     Expect: 3.3e+002  Queries matched: 5
        Mitochondrial ribosomal protein VAR1 - Williopsis mrakii (Yeast) (Hansenula mrakii)
        Observed  Mr(expt)  Mr(calc)   Delta Start   End Miss Ions Peptide
        1184.79   1183.78   1183.67    0.12   281  - 290  0   ---  K.YLIGLSMLFK.G
        1235.73   1234.72   1234.69    0.03   305  - 315  1   ---  K.EKLLFGSLSNK.L
        1381.86   1380.86   1380.74    0.12   335  - 345  1   ---  K.YLNFDININKK.Y
        1795.09   1794.09   1793.95    0.14   133  - 147  1   ---  K.MINNKHTLYYGSLIK.D
        2225.47   2224.46   2224.25    0.21   272  - 290  1   ---  K.ITNELLVNKYLIGLSMLFK.G + Oxidation (M)
        No match to: 807.53, 825.23, 832.62, 835.19, 842.64, 842.64, 847.58, 854.19, 855.19, 856.66, 856.66, 861.20, 864.63,
        865.15, 865.15, 868.66, 870.16, 870.68, 870.68, 871.17, 877.18, 887.14, 887.14, 903.12, 913.55, 973.69, 993.66, 1033.68,
        1033.68, 1037.69, 1045.73, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1106.71, 1107.72, 1107.72,
        1109.68, 1157.77, 1165.77, 1165.77, 1179.79, 1179.79, 1193.81, 1193.81, 1201.81, 1201.81, 1232.80, 1234.88, 1234.88,
        1254.81, 1265.83, 1265.83, 1307.88, 1316.74, 1320.80, 1320.80, 1323.88, 1329.85, 1332.79, 1365.86, 1365.86, 1390.89,
        1434.99, 1476.01, 1476.01, 1658.05, 1708.04, 1708.04, 1717.12, 1742.00, 1792.02, 1792.02, 2211.46, 2211.46, 2384.33


        Matched peptides shown in Bold Red

             1 MTNNKKLYLY KLSSKAMNYS MDKNERNVLY LNKYLHEYNN KGTKLQNSNM
            51 MNSWNNQLYK FNKNEVINTL LTDKLVSKLL VKLFVIKEMG INNPSYLQGS
           101 QMGRRIFINR PKFKHTINTV YINFNYNDTN MKMINNKHTL YYGSLIKDIN
           151 NILGCFNYKN HNNELFNIAT YLSGLYNKKV MIMPNKMKYN YNDNVIFNSS
           201 ISYDLDKYKG GLAGKTYSKL LRDNIPMNNS LSIKNNYMTN IINNNNIKYN
           251 NMISNNSLNI KDIYKSFDIN KITNELLVNK YLIGLSMLFK GKNIKKAGVS
           301 RSIKEKLLFG SLSNKLYRKN SGLLVYKNNN NTTKYLNFDI NINKKYKLNY
           351 MPNHHAISQL SKVNKAKTGV YGISVKLNTI
```

Appendix 4: Technical manuals

i. Technical manual for ACE Kit-WST



ACE Kit - WST Technical Manual 100 tests

Technical Manual (Japanese Version) is available at http://www.dojindo.co.jp/manual/a502.pdf

Angiotensin-converting enzyme (ACE) is one of the key elements responsible for vasopressure action. ACE converts angiotensin-Ⅰ to angiotensin-Ⅱ, a potent vasopressor, in the rennin-angiotensin system, and additionally contributes increasing blood pressure through inactivating bradykinin, a strong antihypertensive peptide. Recently, various functional foods are getting attention due to those ACE inhibition activity.

ACE activity is conventionally determined by the UV reading of the hippuric acid produced from synthetic substrate, Hyppuryl-His-Leu. Because of the assay process is, however, complicated and requires organic solvent, a simple and safe modified method has been developed.

The colorimetric detection system in the ACE Kit-WST can determine the amount of 3-Hyroxybutylic acid (3HB) generated from 3-Hydroxybutylyl-Gly-Gly-Gly with the enzyme method. Since the kit is designed for 96-well microplate assays, it is suitable for multiple sample measurements. Since no organic solvent extraction is required, ACE Kit-WST assay is safe and simple, and gives highly reproducible data.

Fig.1 Principle of the ACE inhibitory activity assay using ACE Kit - WST

Contents of The Kit

- Substrate buffer	2 bottles	- Enzyme A	2 vials
- Enzyme B	2 vials	- Enzyme C	2 vials
- Coenzyme	2 vials	- Indicator solution	2 bottles

Storage

Store the kit at 0-5 °C. The kit is stable for 6 months.

Precaution

- Several kit components are in glass vials. Please handle with care.
- The kit contains two sets. One set is sufficient for 50 tests.
- Multiple measurements such as triplicate is recommended for accurate data.
- If the water solubility of the sample is low, use DMSO or ethanol to dissolve. Then, dilute the solution with an appropriate buffer. The final concentration of organic solvent should be lower than 1 %.
- If the sample solution is acidic, adjust the pH of the sample solution at 5 or higher prior to use for the measurement.
- Ascorbic acid may interfere with the assay. The concentration of the ascorbic acid in the sample solution should be lower than 0.01%w/v.
- If the sample solution contains insoluble materials, remove with centrifuge or filtration prior to use for the measurement.

Required Equipment and Materials

- Microplate reader (450 nm filter)
- 2-20 μl, 20-200 μl, 100-1000 μl pipettes
- multi-channel pipette
- 96-well microplate
- Incubator
- Disposable syringe (1 ml)

Preparation of Working Solution

Enzyme working solution :

Dissolve Enzyme B with 2 ml deionized water to prepare Enzyme B solution. Then add 1.5 ml Enzyme B solution to Enzyme A to prepare Enzyme working solution.

* Enzyme A and B vials are capped under vacuum pressure. Add deionized water or solution through a rubber septum with a syringe, and then remove the septum.

* The Enzyme working solution is stable at -20 °C for 2 weeks. If store in a refrigerator, stable for 3 days.

Indicator working solution :

Dissolve Enzyme C and Coenzyme with 3 ml deionized water each. Add 2.8 ml Enzyme C solution and 2.8 ml Coenzyme solution to Indicator solution to prepare Indicator working solution.

* Enzyme C and Coenzyme vials are capped under vacuum pressure. Add deionized water through a rubber septum with a syringe, and then remove the septum.

* The Indicator working solution is stable at -20 °C for 2 weeks. If store in a refrigerator, stable for 3 days.

A502 ACE Kit WST
Revised March 29 2011

Preparation of Sample Solution

Dilute a sample solution with deionized water.

Dilution ratio : 1(without dilution), 1/5, 1/5^2, 1/5^3, 1/5^4, 1/5^5, 1/5^6

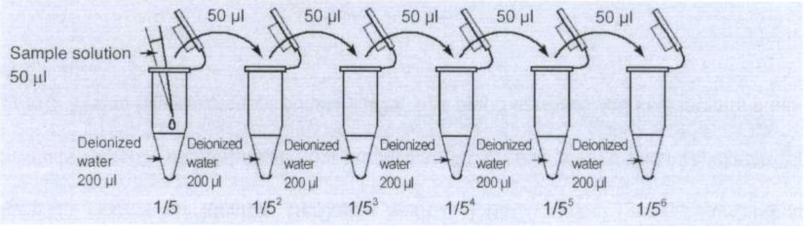


Fig. 2 Preparation of sample solutions

General Procedure for The Assay

See Table and Fig.3

1) Add 20 µl sample solution to a sample well and 20 µl to blank 1 and blank 2 wells.
2) Add 20 µl of Substrate buffer to each well.
3) Add 20 µl deionized water to blank 2 well.
4) Add 20 µl Enzyme working solution to each sample well and blank 1 well.
   * Since the enzymatic reaction starts immediately after the addition of the Enzyme working solution, use a multi-channel pipette to minimize the well-to-well time lag.
5) Incubate at 37°C for 1 hour.
6) Add 200 µl Indicator working solution to each well.
7) Incubate at room temperature for 10 min.
8) Read the absorbance at 450 nm with a microplate reader.
9) ACE inhibitory activity can be calculated by the following equation.

$$\textbf{ACE inhibitory activity (inhibition rate \%)} = [(A_{\text{blank 1}} - A_{\text{sample}}) / (A_{\text{blank 1}} - A_{\text{blank 2}})] \times 100$$

Table. Amount of each solution for sample, blank1 and 2.

	Sample	blank 1	blank 2
Sample solution	20 µl	-	-
Deionized water	-	20 µl	40 µl
Substrate buffer	20 µl	20 µl	20 µl
Enzyme working solution	20 µl	20 µl	
Indicator working solution	200 µl	200 µl	200 µl

blank 1 : positive control (without ACE inhibition)
blank 2 : reagent blank

* Preparation of the sample blank solution (20 µl sample + 240 µl deionized water) will be required for the subtraction of the blank absorbance if the sample solution has visible color.

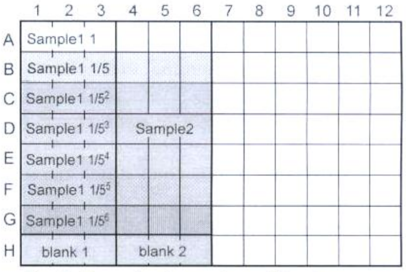


Fig. 3 Example of sample and blank arrangement on a 96-well plate

Determination of IC_{50} (50% inhibitory concentration)

- Prepare an inhibition curve with using the sample concentration for X axis and ACE inhibitory activity for Y axis. Typical inhibition curve is shown in Fig. 4.
- Determine the concentration of the sample solution that gives 50% ACE inhibitory activity as indicated in the Fig. 4 .
- Since the total volume of the inhibition assay is 60 µl (first step of the assay), the original sample is diluted in 3 times in the reaction. Therefore, the concentration of the sample gives 50% inhibition is one third of the concentration determined by the inhibition curve.

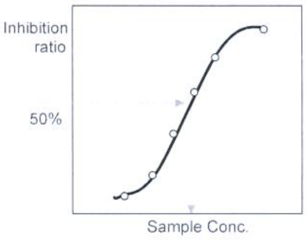
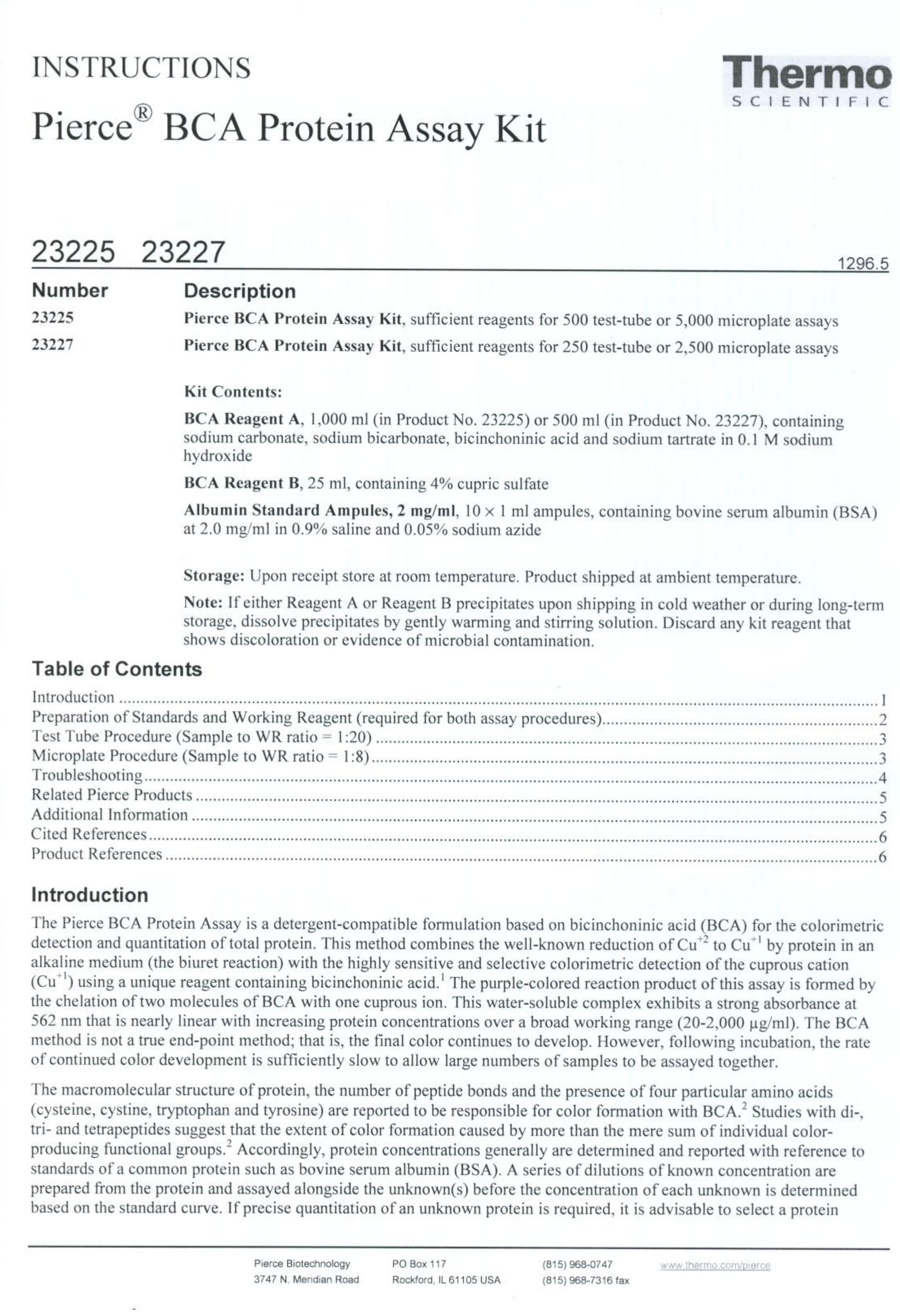


Fig.4 Inhibition curve

References

1. Le Hoang Lam, T. Shimamura, K. Sakaguchi, K. Noguchi, M Ishiyama, Y. Fujimura and H. Ukeda, *Anal. Biochem.*, **2007**, *364*, 104.
2. Le Hoang Lam, T. Shimamura, S. Manabe, M Ishiyama and H. Ukeda, *Anal. Sci.*, **2008**, 24, 1057.

ii. Technical manual for Pierce BCA Protein Assay Kit



INSTRUCTIONS

Pierce® BCA Protein Assay Kit

Thermo SCIENTIFIC

23225 23227

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Number	Description
23225	**Pierce BCA Protein Assay Kit**, sufficient reagents for 500 test-tube or 5,000 microplate assays
23227	**Pierce BCA Protein Assay Kit**, sufficient reagents for 250 test-tube or 2,500 microplate assays

Kit Contents:

BCA Reagent A, 1,000 ml (in Product No. 23225) or 500 ml (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide

BCA Reagent B, 25 ml, containing 4% cupric sulfate

Albumin Standard Ampules, 2 mg/ml, 10 × 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store at room temperature. Product shipped at ambient temperature.

Note: If either Reagent A or Reagent B precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solution. Discard any kit reagent that shows discoloration or evidence of microbial contamination.

Table of Contents

Introduction

The Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.[1] The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation caused by more than the mere sum of individual color-producing functional groups.[2] Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. If precise quantitation of an unknown protein is required, it is advisable to select a protein

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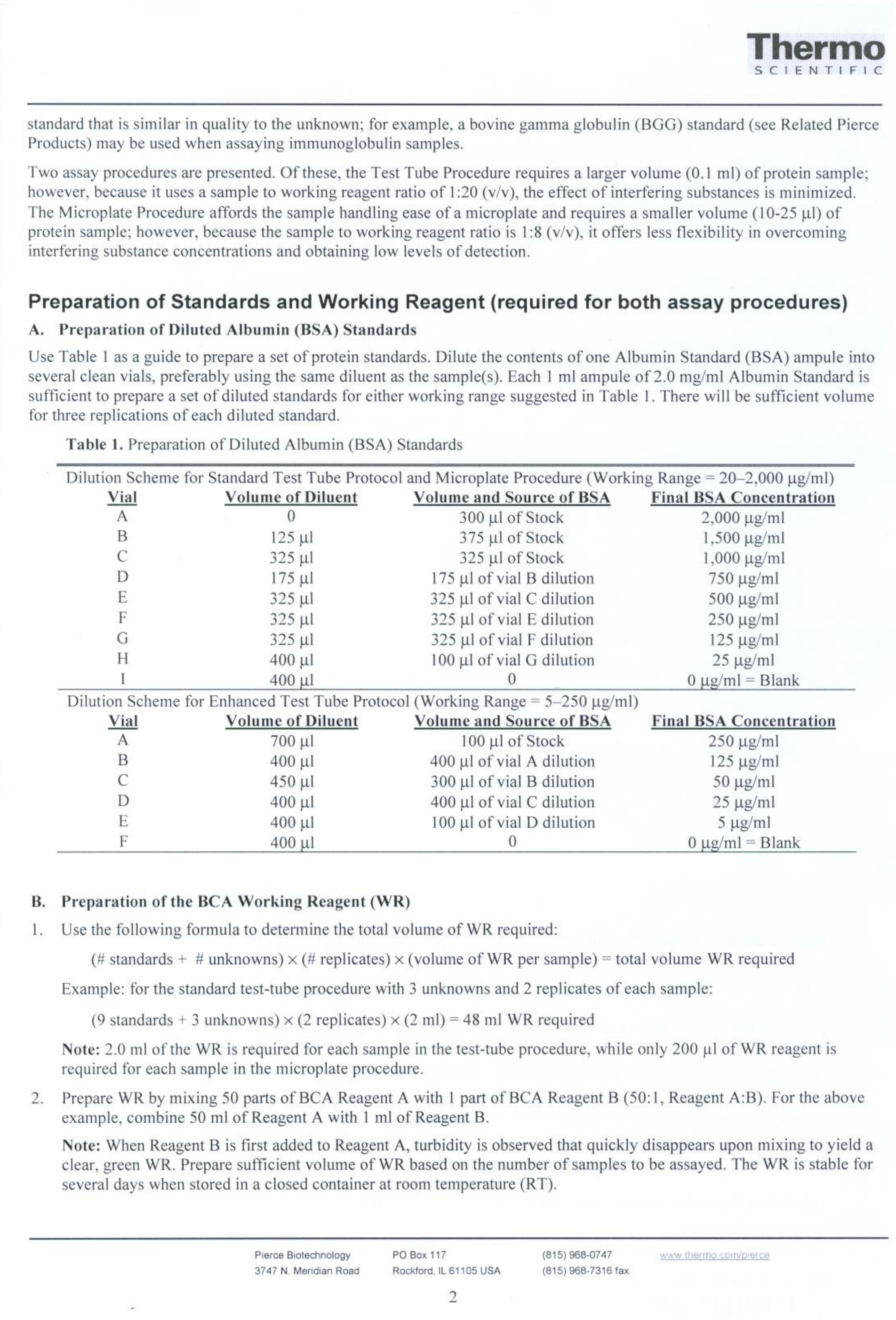

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standard that is similar in quality to the unknown; for example, a bovine gamma globulin (BGG) standard (see Related Pierce Products) may be used when assaying immunoglobulin samples.

Two assay procedures are presented. Of these, the Test Tube Procedure requires a larger volume (0.1 ml) of protein sample; however, because it uses a sample to working reagent ratio of 1:20 (v/v), the effect of interfering substances is minimized. The Microplate Procedure affords the sample handling ease of a microplate and requires a smaller volume (10-25 µl) of protein sample; however, because the sample to working reagent ratio is 1:8 (v/v), it offers less flexibility in overcoming interfering substance concentrations and obtaining low levels of detection.

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	700 µl	100 µl of Stock	250 µg/ml
B	400 µl	400 µl of vial A dilution	125 µg/ml
C	450 µl	300 µl of vial B dilution	50 µg/ml
D	400 µl	400 µl of vial C dilution	25 µg/ml
E	400 µl	100 µl of vial D dilution	5 µg/ml
F	400 µl	0	0 µg/ml = Blank

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

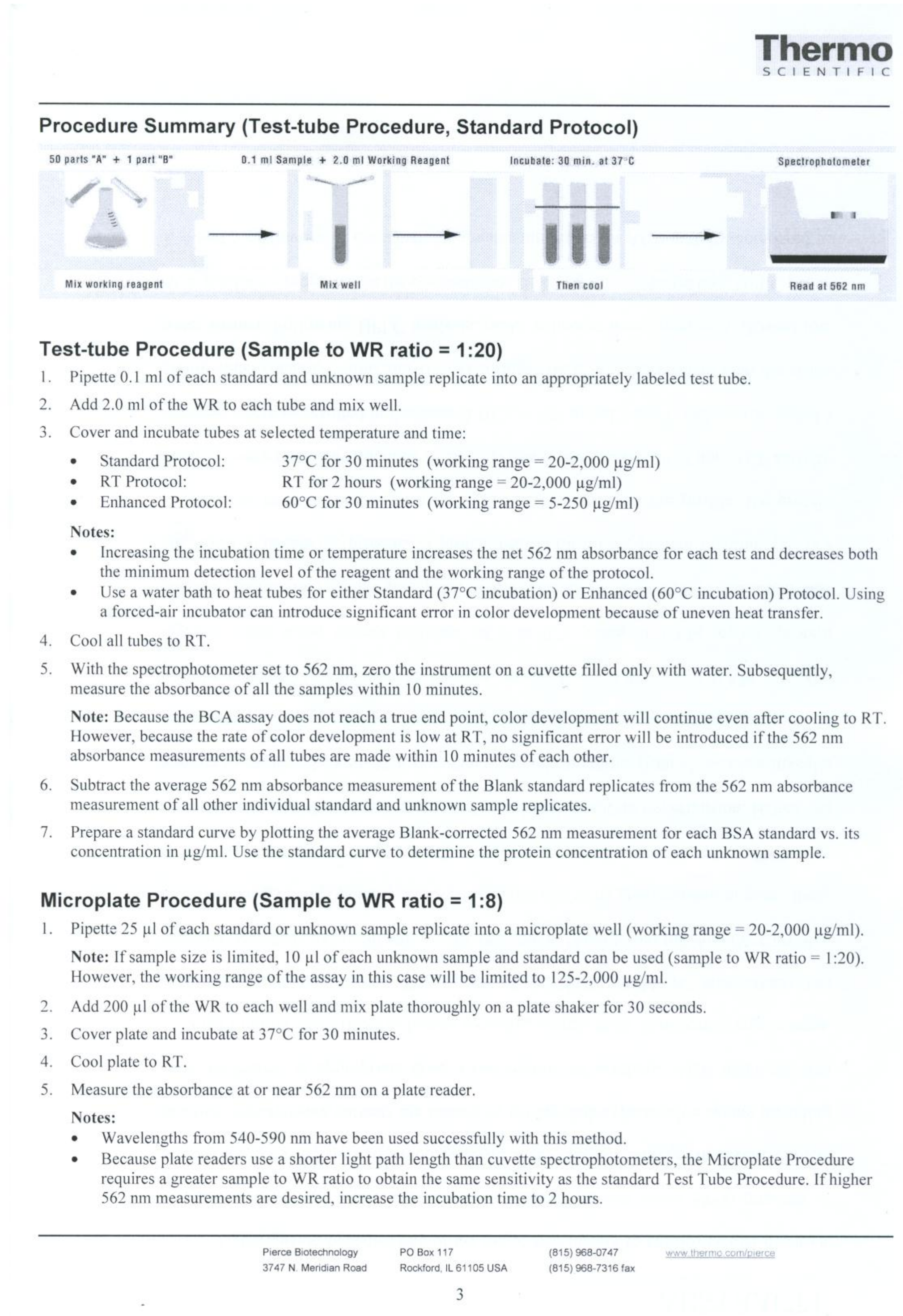
   Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

   (9 standards + 3 unknowns) × (2 replicates) × (2 ml) = 48 ml WR required

   Note: 2.0 ml of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.

   Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).



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Procedure Summary (Test-tube Procedure, Standard Protocol)

Test-tube Procedure (Sample to WR ratio = 1:20)

1. Pipette 0.1 ml of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0 ml of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:

• Standard Protocol:	37°C for 30 minutes (working range = 20-2,000 μg/ml)
• RT Protocol:	RT for 2 hours (working range = 20-2,000 μg/ml)
• Enhanced Protocol:	60°C for 30 minutes (working range = 5-250 μg/ml)

   Notes:
   - Increasing the incubation time or temperature increases the net 562 nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.

4. Cool all tubes to RT.
5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562 nm absorbance measurements of all tubes are made within 10 minutes of each other.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in μg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

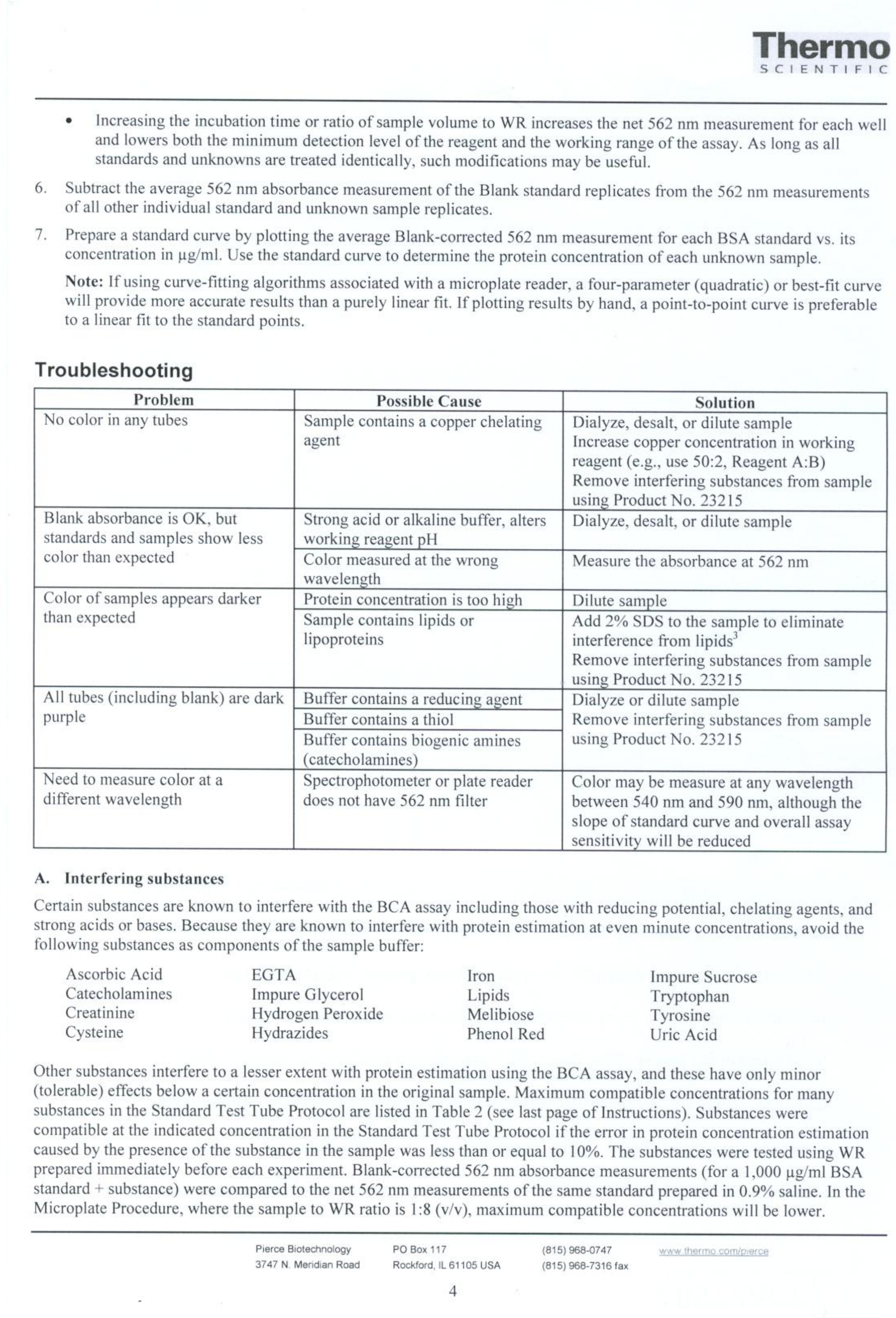
Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25 μl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 μg/ml).

   Note: If sample size is limited, 10 μl of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2,000 μg/ml.

2. Add 200 μl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT.
5. Measure the absorbance at or near 562 nm on a plate reader.

   Notes:
   - Wavelengths from 540-590 nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase the incubation time to 2 hours.



- Increasing the incubation time or ratio of sample volume to WR increases the net 562 nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze, desalt, or dilute sample Increase copper concentration in working reagent (e.g., use 50:2, Reagent A:B) Remove interfering substances from sample using Product No. 23215
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt, or dilute sample
	Color measured at the wrong wavelength	Measure the absorbance at 562 nm
Color of samples appears darker than expected	Protein concentration is too high	Dilute sample
	Sample contains lipids or lipoproteins	Add 2% SDS to the sample to eliminate interference from lipids[3] Remove interfering substances from sample using Product No. 23215
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample Remove interfering substances from sample using Product No. 23215
	Buffer contains a thiol	
	Buffer contains biogenic amines (catecholamines)	
Need to measure color at a different wavelength	Spectrophotometer or plate reader does not have 562 nm filter	Color may be measure at any wavelength between 540 nm and 590 nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with the BCA assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

- Ascorbic Acid
- Catecholamines
- Creatinine
- Cysteine
- EGTA
- Impure Glycerol
- Hydrogen Peroxide
- Hydrazides
- Iron
- Lipids
- Melibiose
- Phenol Red
- Impure Sucrose
- Tryptophan
- Tyrosine
- Uric Acid

Other substances interfere to a lesser extent with protein estimation using the BCA assay, and these have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page of Instructions). Substances were compatible at the indicated concentration in the Standard Test Tube Protocol if the error in protein concentration estimation caused by the presence of the substance in the sample was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. Blank-corrected 562 nm absorbance measurements (for a 1,000 µg/ml BSA standard + substance) were compared to the net 562 nm measurements of the same standard prepared in 0.9% saline. In the Microplate Procedure, where the sample to WR ratio is 1:8 (v/v), maximum compatible concentrations will be lower.