Figure 1

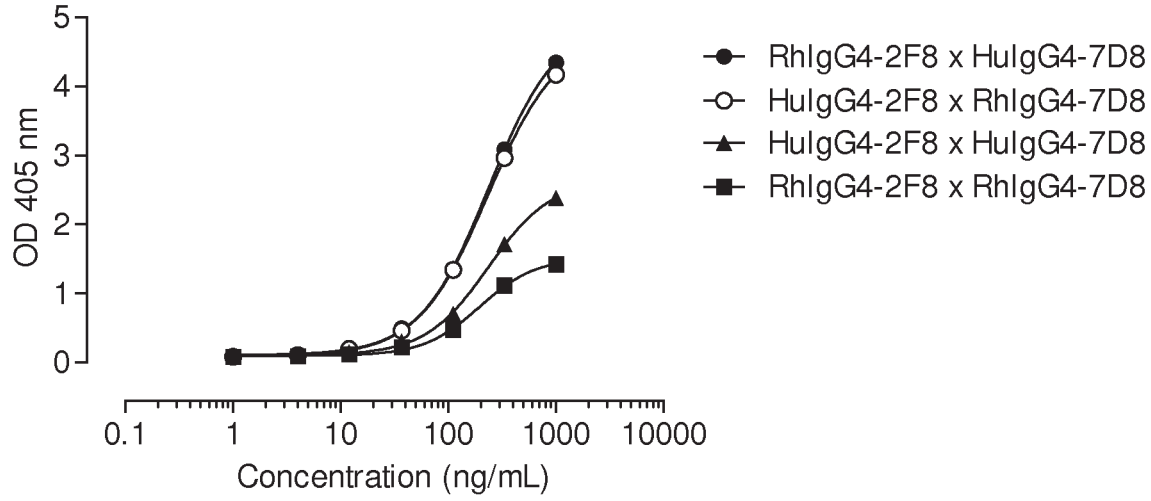

OD 405 nm
5
4
3
2
1
0
0.1
1
10
100
1000
10000
Concentration (ng/mL)
RhIgG4-2F8 x HuIgG4-7D8
HuIgG4-2F8 x RhIgG4-7D8
HuIgG4-2F8 x HuIgG4-7D8
RhIgG4-2F8 x RhIgG4-7D8

Figure 2

Species (common name)	Isotype	Amino-acid position*																					
		Core-hinge					CH3-CH3 interface																
		226	227	228	229	230	347	349	350	351	366	368	370	392	394	395	397	398	399	405	407	409
Homo sapiens	IgG1	C	P	P	C	P	Q	Y	T	L	T	L	K	K	T	P	V	L	D	F	Y	K
	IgG2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-
	IgG3	-	-	R	-	-	-	-	-	-	-	-	-	N	-	-	M	-	-	-	-	-
	IgG4	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
Macaca mulatta	IgG1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Rhesus Monkey)	IgG2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgG3	-	-	-	-	-	-	-	I	-	-	-	T	-	-	-	-	-	-	-	-	-
	IgG4 (In)**	-	-	-	-	-	-	-	I	-	-	-	T	-	-	-	-	-	-	L	-	-
	IgG4 (Ch)***	-	-	A	-	-	-	-	I	-	-	-	T	-	-	-	-	-	-	L	-	-

*EU numbering; **In, Indian; ***Ch, Chinese

Figure 3

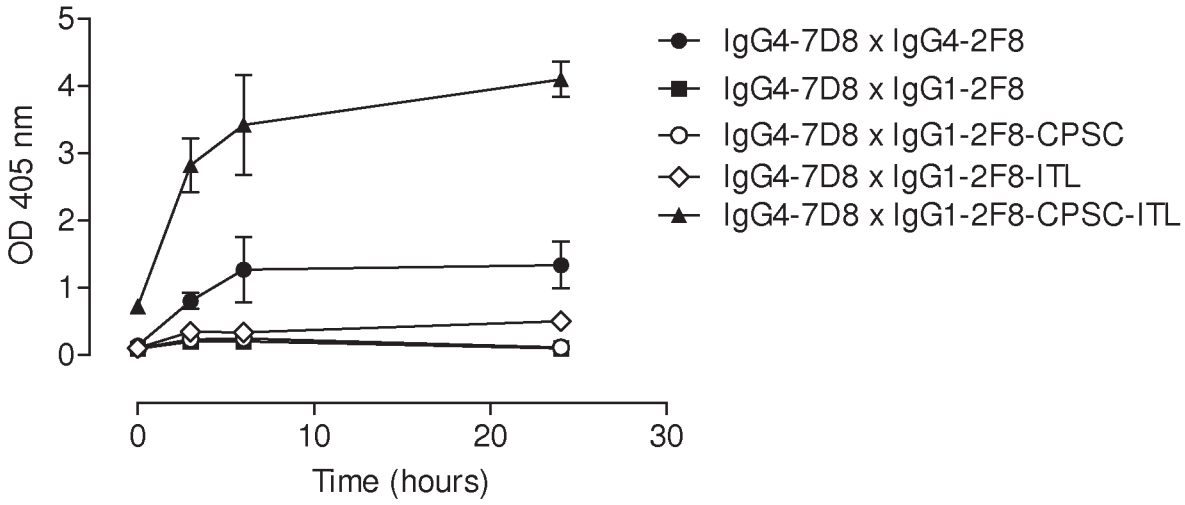

OD 405 nm
0
1
2
3
4
5
0
10
20
30
Time (hours)
IgG4-7D8 x IgG4-2F8
IgG4-7D8 x IgG1-2F8
IgG4-7D8 x IgG1-2F8-CPSC
IgG4-7D8 x IgG1-2F8-ITL
IgG4-7D8 x IgG1-2F8-CPSC-ITL

Figure 4

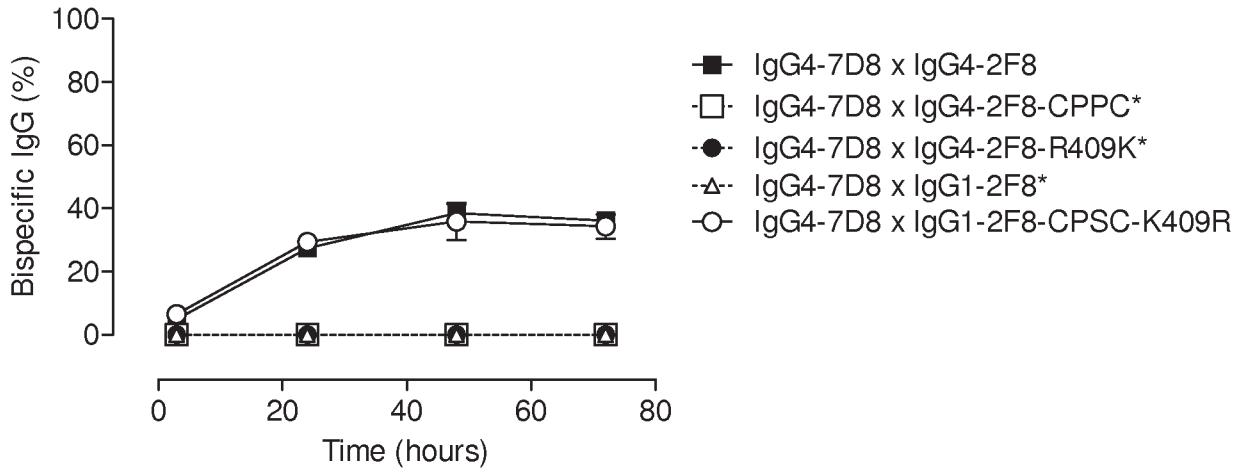

Bispecific IgG (%)
100
80
60
40
20
0
0
20
40
60
80
Time (hours)
IgG4-7D8 x IgG4-2F8
IgG4-7D8 x IgG4-2F8-CPPC*
IgG4-7D8 x IgG4-2F8-R409K*
IgG4-7D8 x IgG1-2F8*
IgG4-7D8 x IgG1-2F8-CPSC-K409R

Figure 5

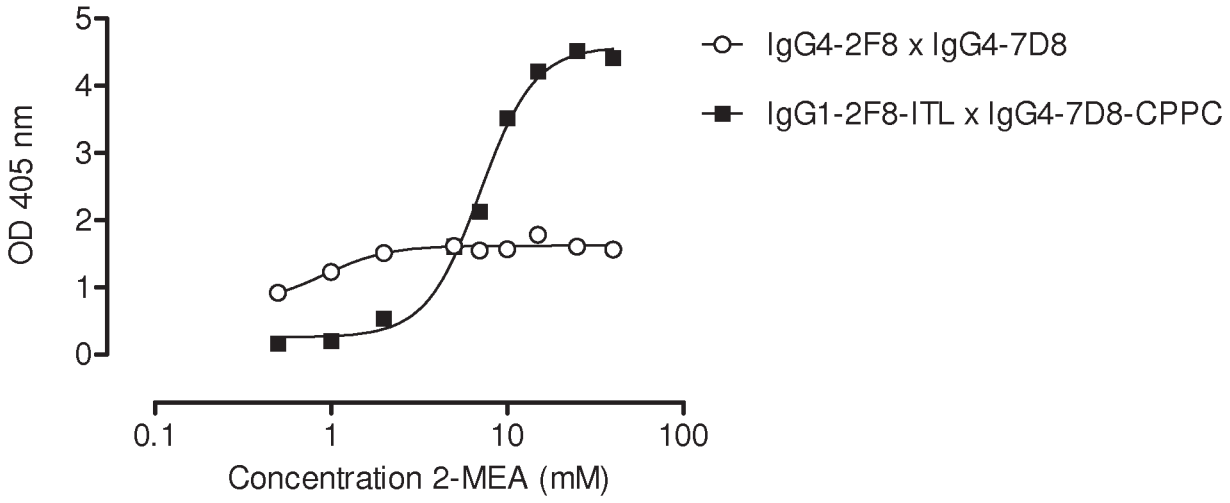

OD 405 nm
5
4
3
2
1
0
0.1
1
10
100
Concentration 2-MEA (mM)
IgG4-2F8 x IgG4-7D8
IgG1-2F8-ITL x IgG4-7D8-CPPC

Figure 6

A

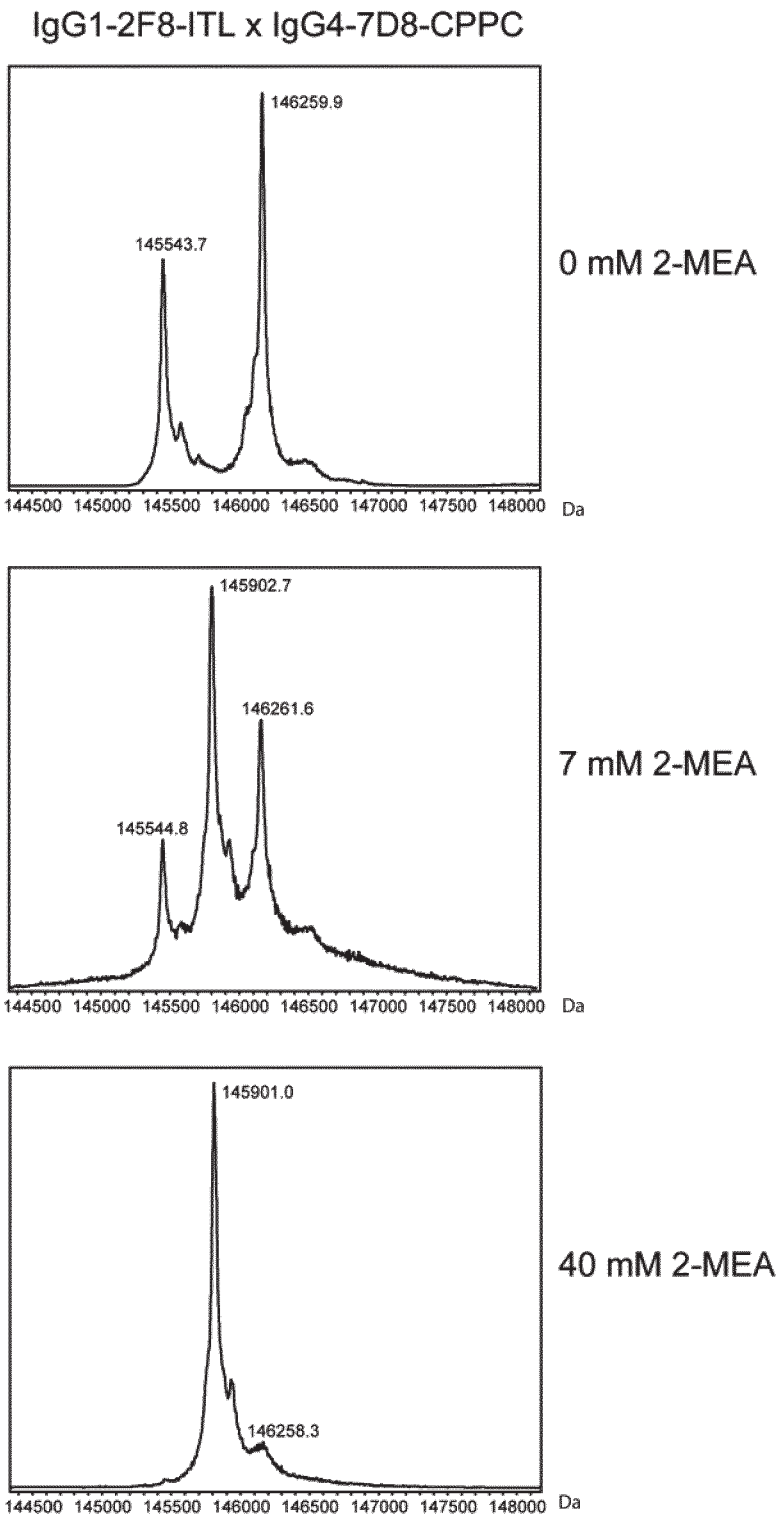

IgG1-2F8-ITL x IgG4-7D8-CPPC
146259.9
145543.7
0 mM 2-MEA
144500 145000 145500 146000 146500 147000 147500 148000 Da
145902.7
146261.6
145544.8
7 mM 2-MEA
144500 145000 145500 146000 146500 147000 147500 148000 Da
145901.0
146258.3
40 mM 2-MEA
144500 145000 145500 146000 146500 147000 147500 148000 Da

Figure 6 (continued)

B

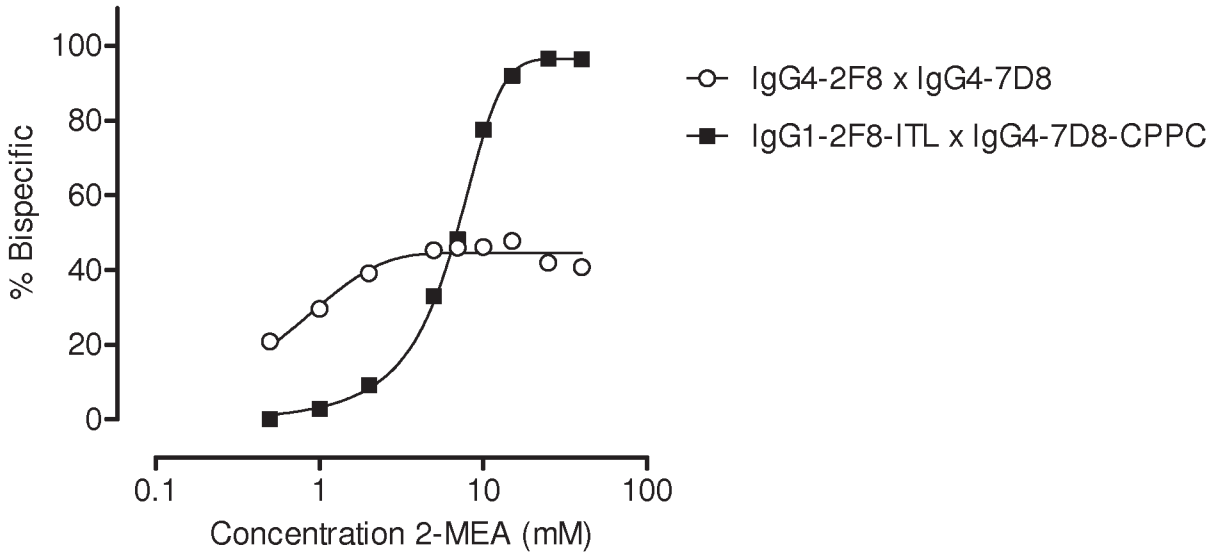
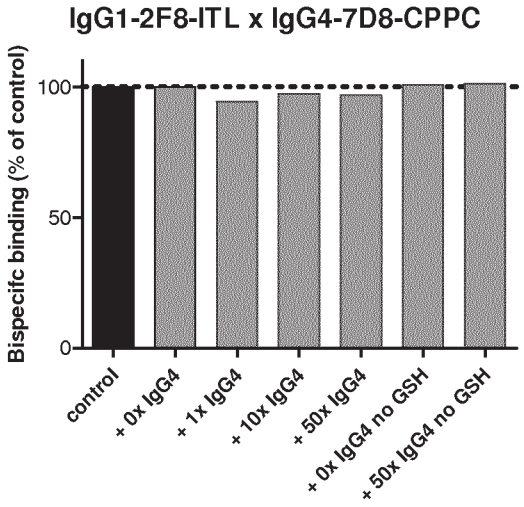


Figure 7

A


IgG1-2F8-ITL x IgG4-7D8-CPPC
Bispecifc binding (% of control)
100
50
0
control
+ 0x IgG4
+ 1x IgG4
+ 10x IgG4
+ 50x IgG4
+ 0x IgG4 no GSH
+ 50x IgG4 no GSH

B

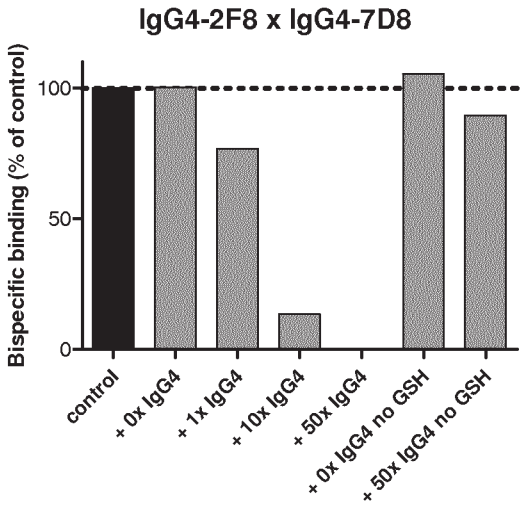
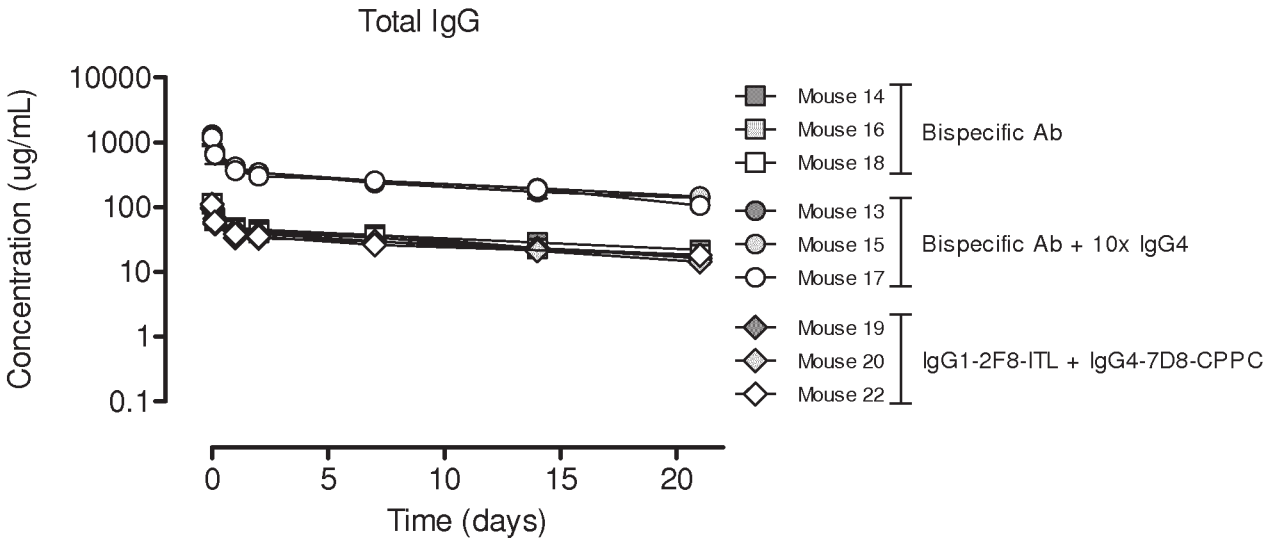

IgG4-2F8 x IgG4-7D8
Bispecific binding (% of control)
100
50
0
control
+ 0x IgG4
+ 1x IgG4
+ 10x IgG4
+ 50x IgG4
+ 0x IgG4 no GSH
+ 50x IgG4 no GSH

Figure 8

A


Total IgG
Concentration (ug/mL)
10000
1000
100
10
1
0.1
0
5
10
15
20
Time (days)
Mouse 14
Mouse 16
Mouse 18
Bispecific Ab
Mouse 13
Mouse 15
Mouse 17
Bispecific Ab + 10x IgG4
Mouse 19
Mouse 20
Mouse 22
IgG1-2F8-ITL + IgG4-7D8-CPPC

B

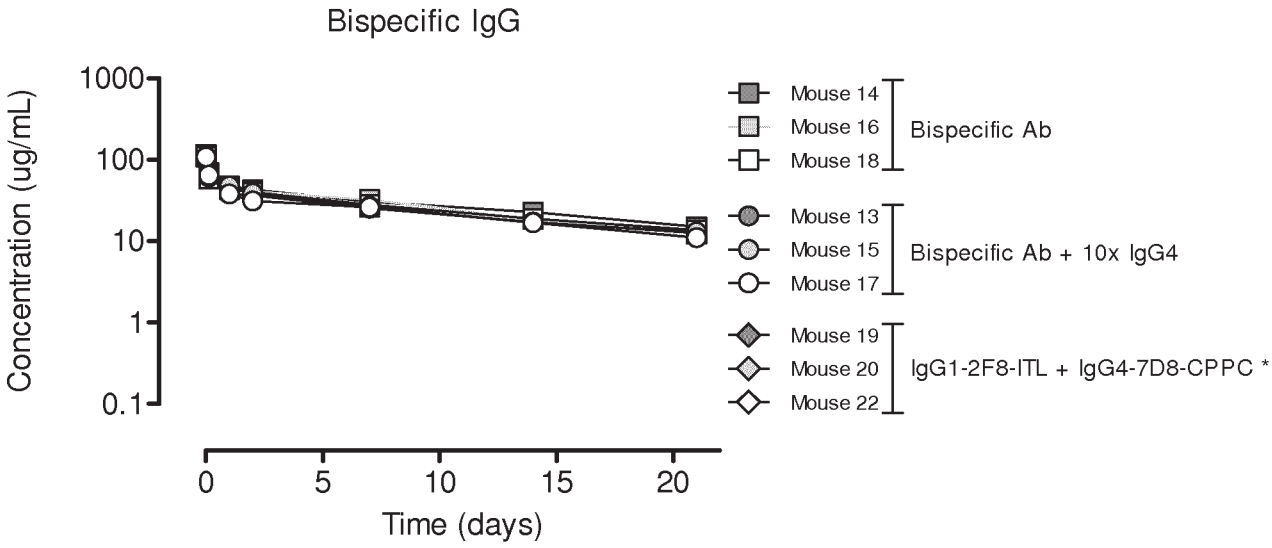
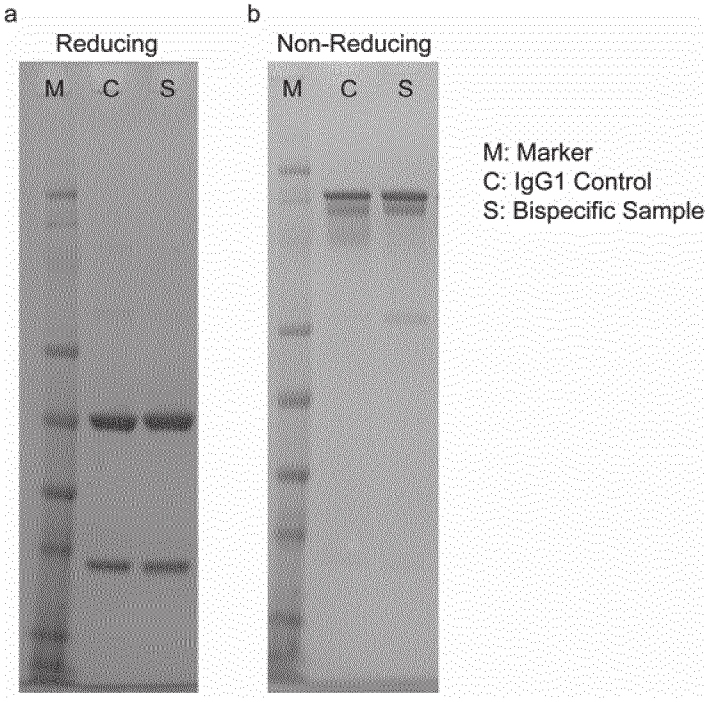
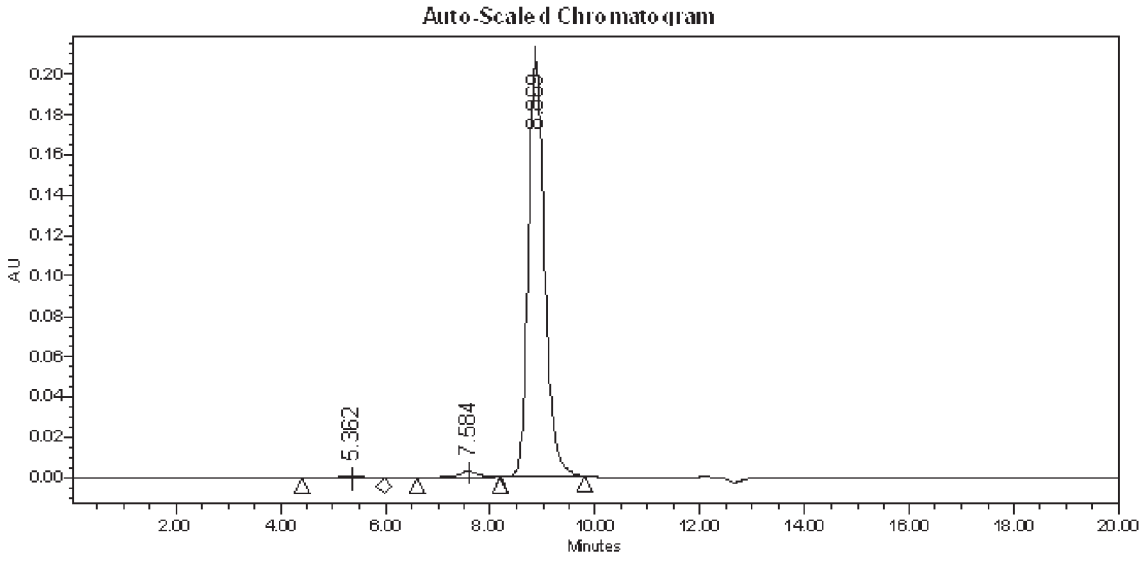

Bispecific IgG
Concentration (ug/mL)
1000
100
10
1
0.1
0
5
10
15
20
Time (days)
Mouse 14
Mouse 16
Mouse 18
Bispecific Ab
Mouse 13
Mouse 15
Mouse 17
Bispecific Ab + 10x IgG4
Mouse 19
Mouse 20
Mouse 22
IgG1-2F8-ITL + IgG4-7D8-CPPC *

Figure 9

A



B

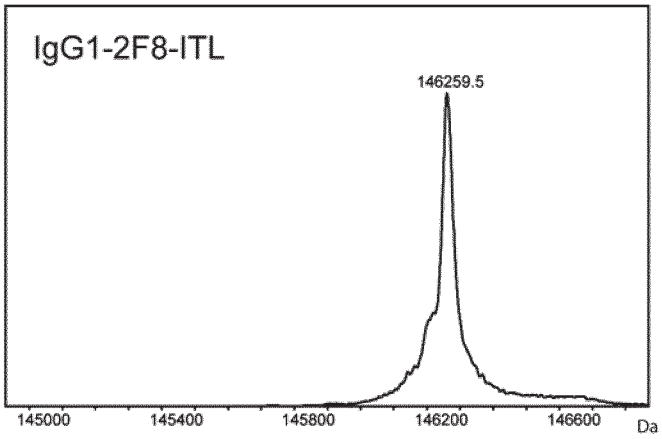


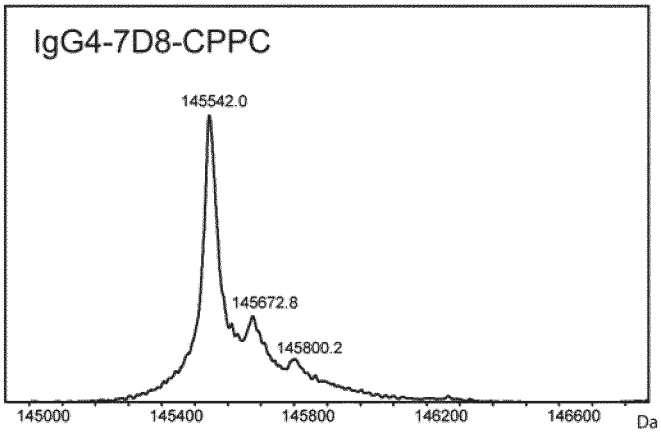
Peak results:

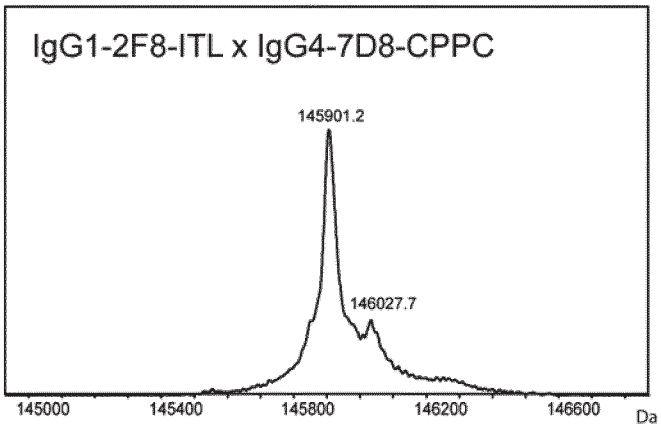
	RT (min)	Area	Height	% Height
1	5.362	18309	448	0.21
2	7.584	82197	2876	1.36
3	8.869	4431069	207416	98.42

Figure 9 (continued)

C



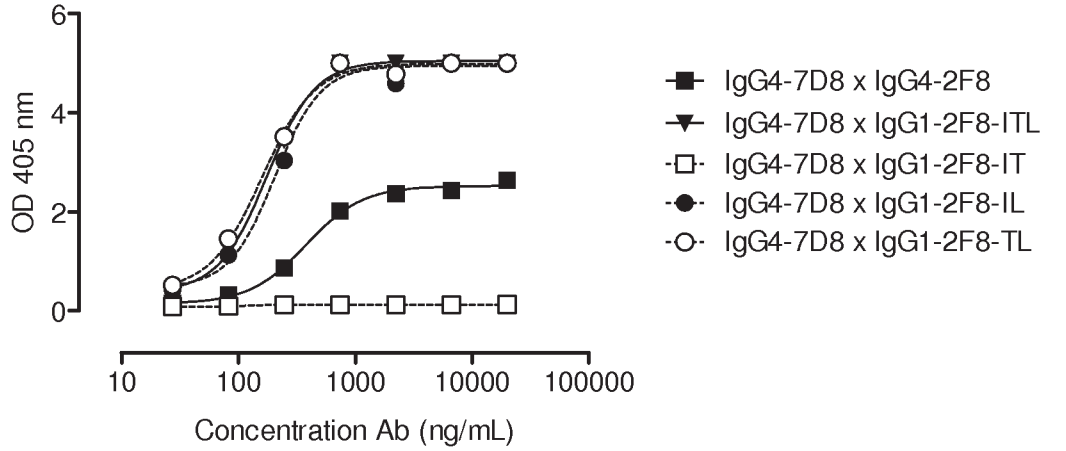




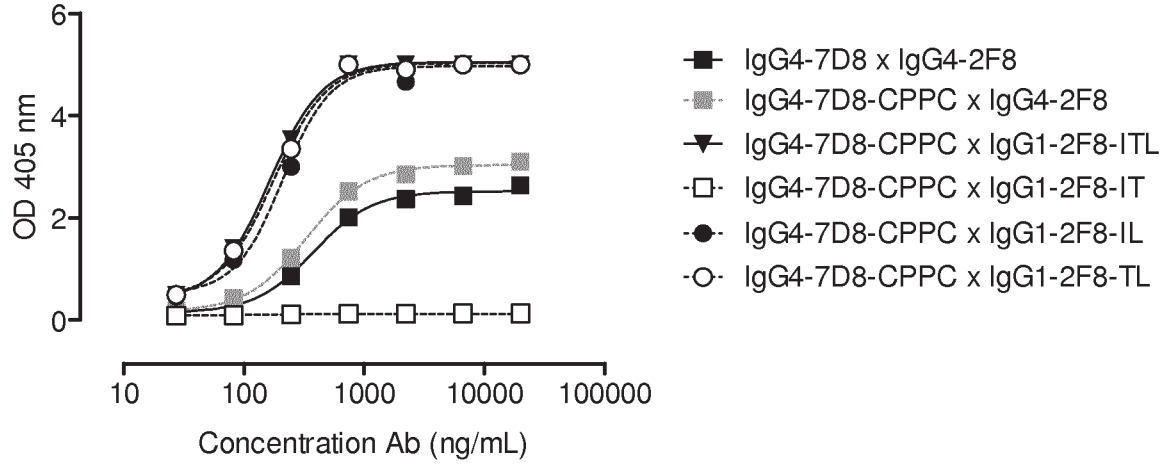
	Measured	Measured	Measured
IgG1-2F8-ITL	146259.5		
IgG4-7D8-CPPC	145542.0	145672.8	145800.2
IgG1-2F8-ITL x IgG4-7D8-	145901.2	146027.7	

Figure 10

(A)



(B)



(C)

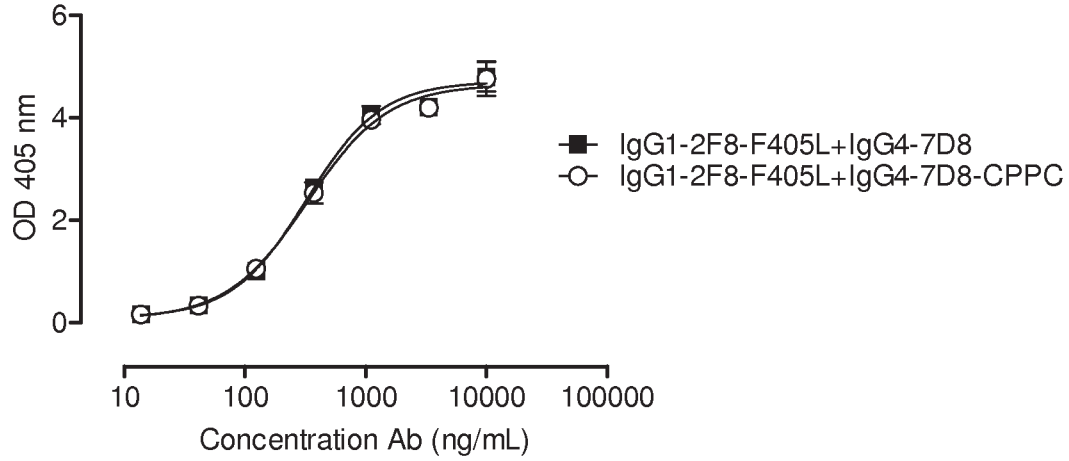


Figure 11

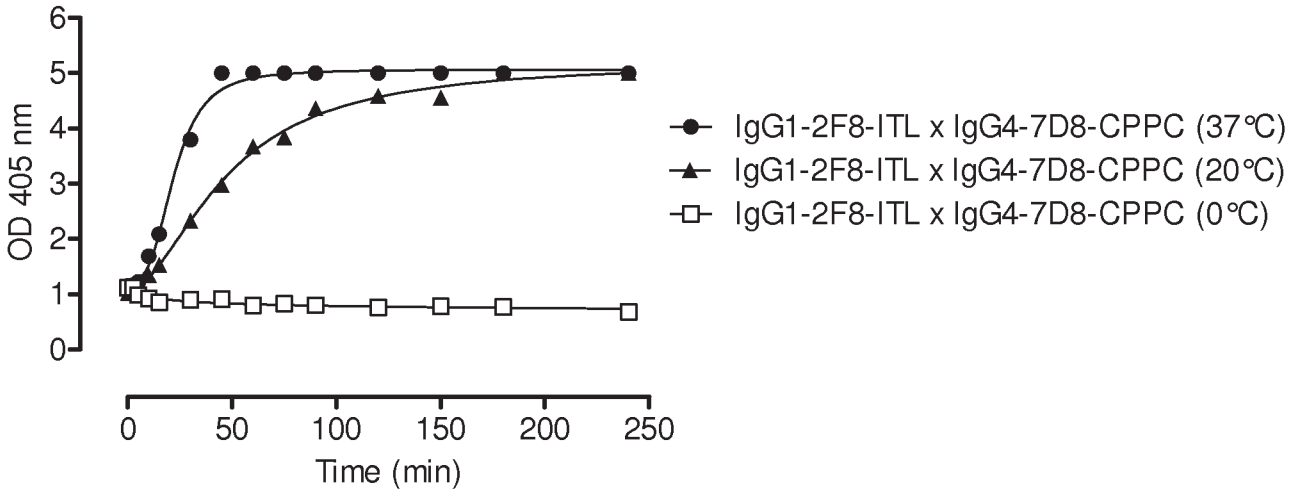

OD 405 nm
6
5
4
3
2
1
0
0
50
100
150
200
250
Time (min)
IgG1-2F8-ITL x IgG4-7D8-CPPC (37°C)
IgG1-2F8-ITL x IgG4-7D8-CPPC (20°C)
IgG1-2F8-ITL x IgG4-7D8-CPPC (0°C)

Figure 12

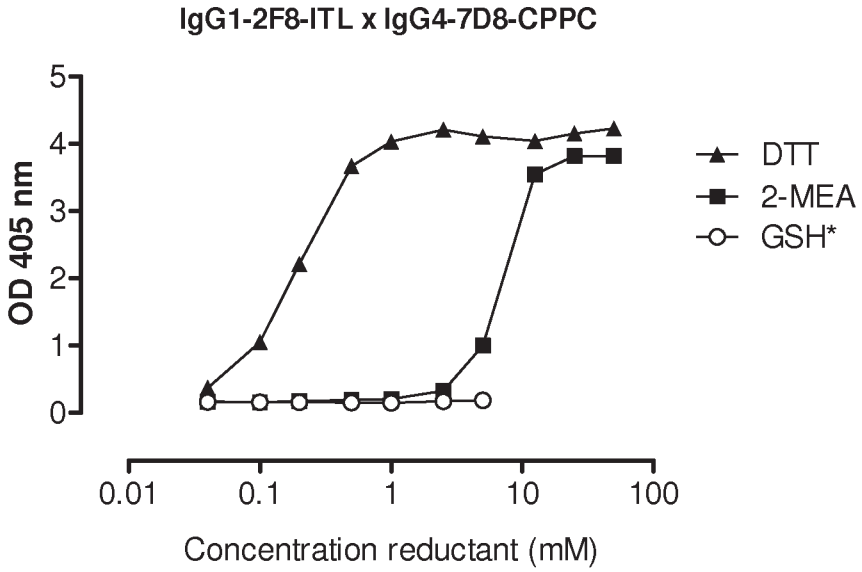
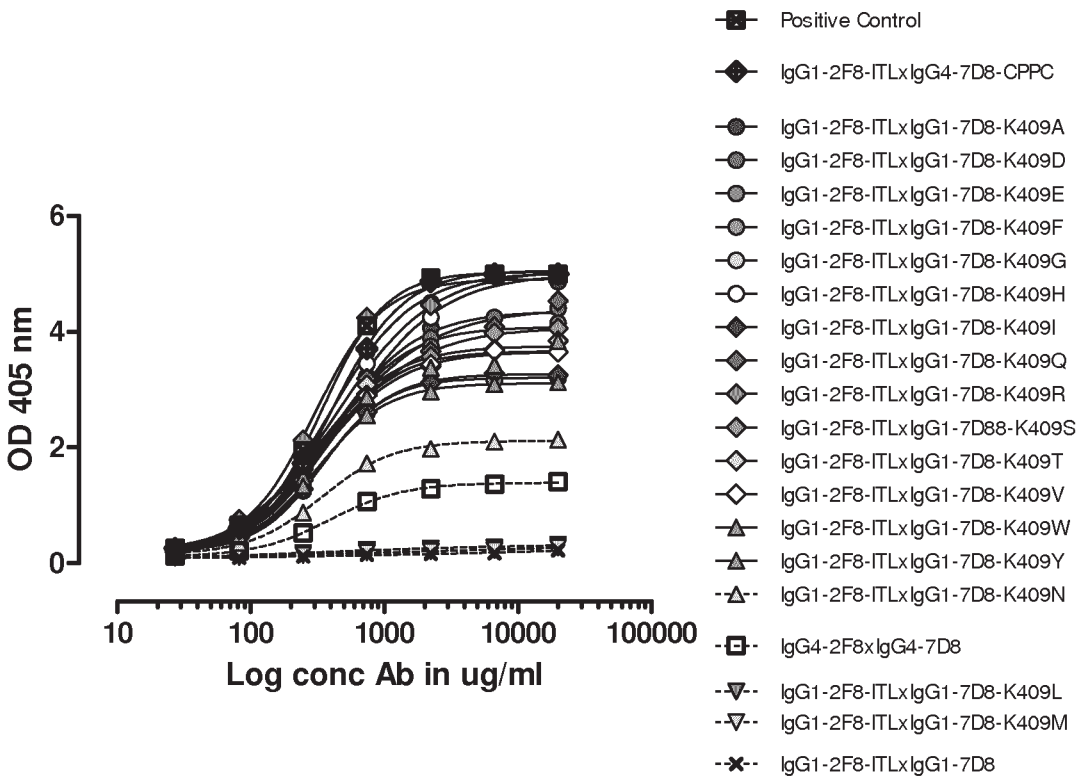

IgG1-2F8-ITL x IgG4-7D8-CPPC
OD 405 nm
5
4
3
2
1
0
0.01
0.1
1
10
100
Concentration reductant (mM)
DTT
2-MEA
GSH*

Figure 13

A


OD 405 nm
6
4
2
0
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG1-2F8-ITLxIgG4-7D8-CPPC
IgG1-2F8-ITLxIgG1-7D8-K409A
IgG1-2F8-ITLxIgG1-7D8-K409D
IgG1-2F8-ITLxIgG1-7D8-K409E
IgG1-2F8-ITLxIgG1-7D8-K409F
IgG1-2F8-ITLxIgG1-7D8-K409G
IgG1-2F8-ITLxIgG1-7D8-K409H
IgG1-2F8-ITLxIgG1-7D8-K409I
IgG1-2F8-ITLxIgG1-7D8-K409Q
IgG1-2F8-ITLxIgG1-7D8-K409R
IgG1-2F8-ITLxIgG1-7D88-K409S
IgG1-2F8-ITLxIgG1-7D8-K409T
IgG1-2F8-ITLxIgG1-7D8-K409V
IgG1-2F8-ITLxIgG1-7D8-K409W
IgG1-2F8-ITLxIgG1-7D8-K409Y
IgG1-2F8-ITLxIgG1-7D8-K409N
IgG4-2F8xIgG4-7D8
IgG1-2F8-ITLxIgG1-7D8-K409L
IgG1-2F8-ITLxIgG1-7D8-K409M
IgG1-2F8-ITLxIgG1-7D8

B

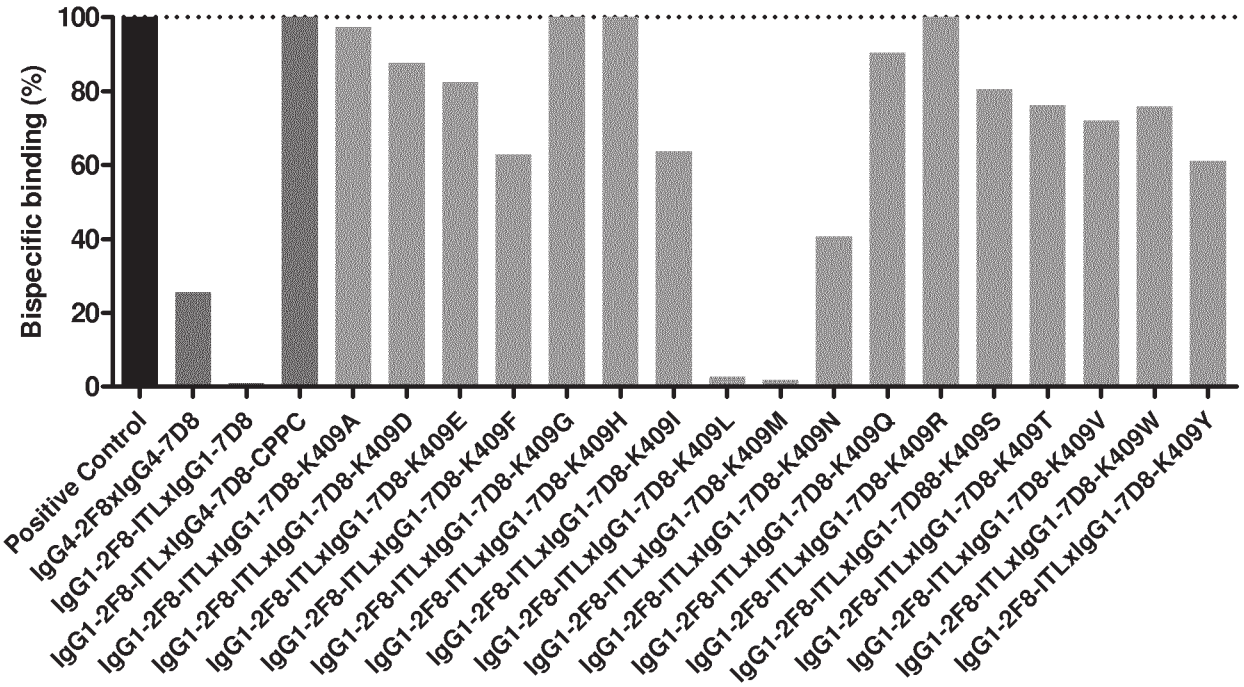

Bispecific binding (%)
100
80
60
40
20
0
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8-ITLxIgG1-7D8
IgG1-2F8-ITLxIgG4-7D8-CPPC
IgG1-2F8-ITLxIgG1-7D8-K409A
IgG1-2F8-ITLxIgG1-7D8-K409D
IgG1-2F8-ITLxIgG1-7D8-K409E
IgG1-2F8-ITLxIgG1-7D8-K409F
IgG1-2F8-ITLxIgG1-7D8-K409G
IgG1-2F8-ITLxIgG1-7D8-K409H
IgG1-2F8-ITLxIgG1-7D8-K409I
IgG1-2F8-ITLxIgG1-7D8-K409L
IgG1-2F8-ITLxIgG1-7D8-K409M
IgG1-2F8-ITLxIgG1-7D8-K409N
IgG1-2F8-ITLxIgG1-7D8-K409Q
IgG1-2F8-ITLxIgG1-7D8-K409R
IgG1-2F8-ITLxIgG1-7D8-K409S
IgG1-2F8-ITLxIgG1-7D88-K409T
IgG1-2F8-ITLxIgG1-7D8-K409V
IgG1-2F8-ITLxIgG1-7D8-K409W
IgG1-2F8-ITLxIgG1-7D8-K409Y

Figure 14

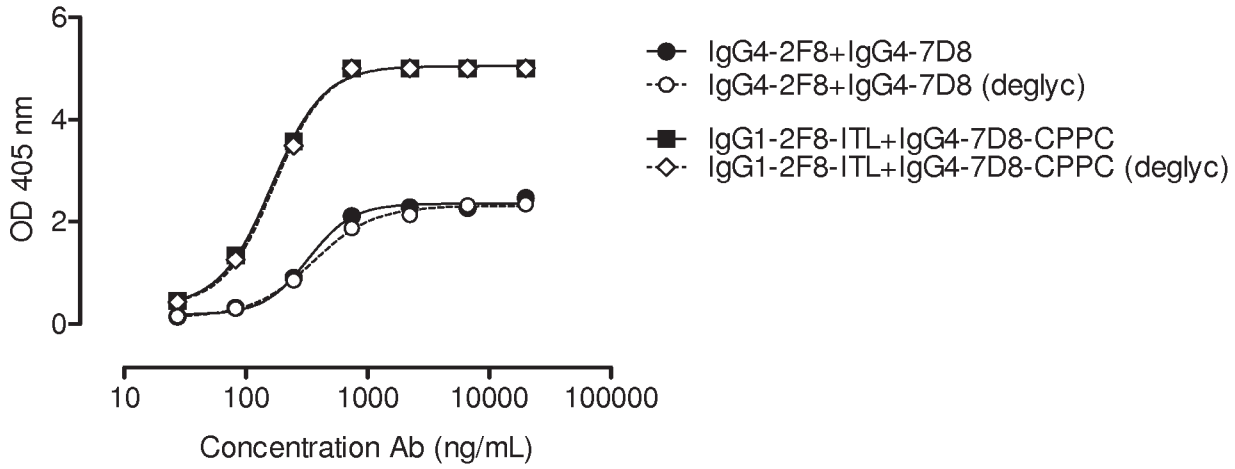
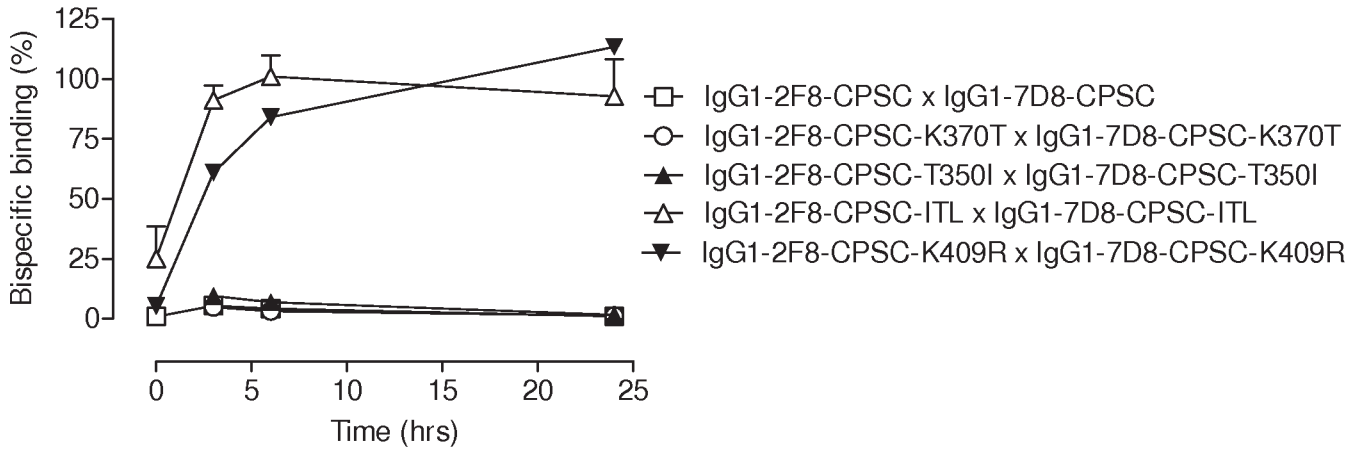
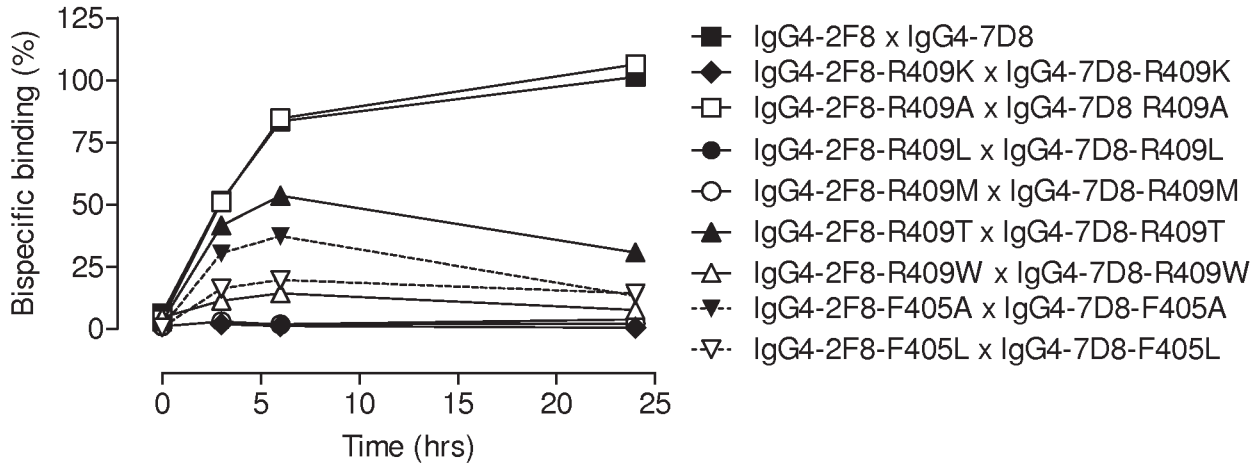

6
4
2
0
OD 405 nm
10
100
1000
10000
100000
Concentration Ab (ng/mL)
IgG4-2F8+IgG4-7D8
IgG4-2F8+IgG4-7D8 (deglyc)
IgG1-2F8-ITL+IgG4-7D8-CPPC
IgG1-2F8-ITL+IgG4-7D8-CPPC (deglyc)

Figure 15

A



B



C

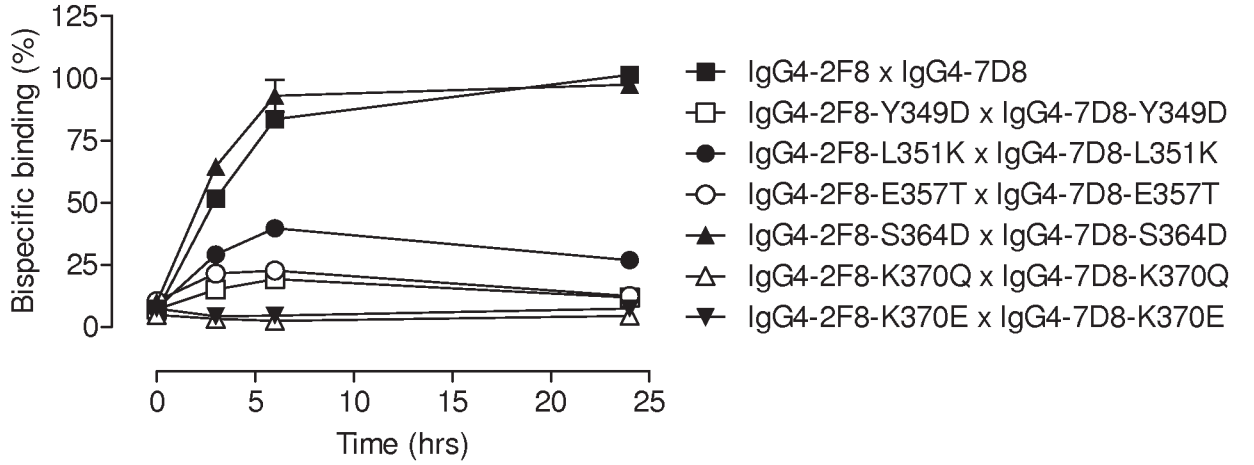
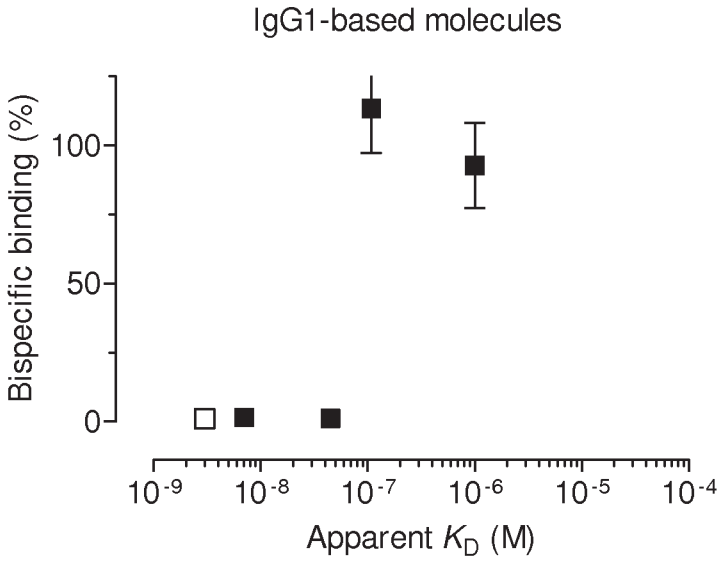


Figure 15 (continued)

D



E

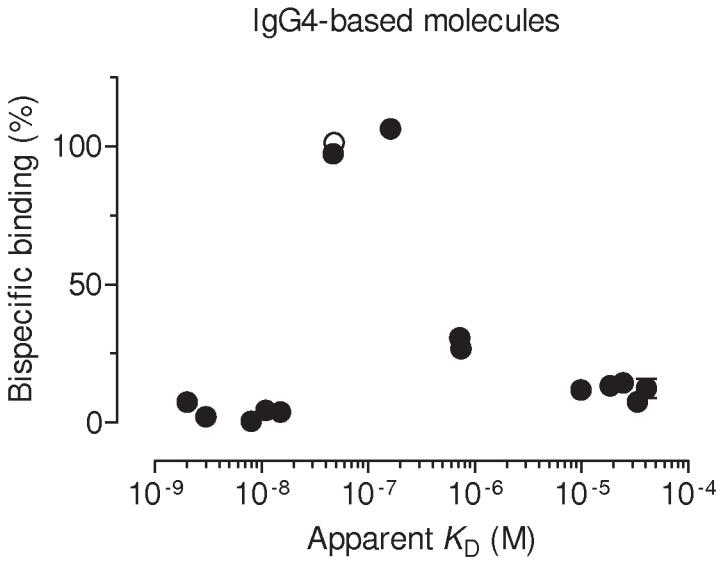


Figure 16

```
EU                                                                                                                 1
                 1         2         3           4         5           6         7         8         9         0
        12345678901234567890123456789012345  678901234567890123  45678901234567890123456789012345678901234567890            12
2F8-G1  QVQLVESGGGVVQPGRSLRLSCAASGFTFSTYGMH--WVRQAPGKGLEWVAVIWD--DGSYKYYGDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDGITMVRGVMKDYFDY
2F8-G4  QVQLVESGGGVVQPGRSLRLSCAASGFTFSTYGMH--WVRQAPGKGLEWVAVIWD--DGSYKYYGDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDGITMVRGVMKDYFDY
KABAT                                                                                                                  1
                 1         2         3           4         5            6         7         8            9         0
        1234567890123456789012345678901234555678901234567890122223456789012345678901234567890122223456789012345678900000000012
                                           AB                 ABC                              ABC                  ABCDEFGH

EU      1      1          1         1           1         1              1          1          1         1            2
        0      1          2         3           4         5              6          7          8         9            0
        345678901234567 89012345678901234  5678901234567890123456 78    90123456 7890123456 7890123456789012345  6 7890
2F8-G1  WGQGTLVTVSS-----ASTKGPSVFPLAPSSKS--TSGGTAALGCLVKDYFPEPVTV-SW----NSGALTSG-VHTFPAVLQS-SGLYSLSSVVTVPSSSLGT--Q-TYIC
2F8-G4  WGQGTLVTVSS-----ASTKGPSVFPLAPCSRS--TSESTAALGCLVKDYFPEPVTV-SW----NSGALTSG-VHTFPAVLQS-SGLYSLSSVVTVPSSSLGT--K-TYTC
KABAT   1      1              1         1         1         1         1         1         1         1         2
        0      1              2         3         4         5         6         7         8         9         0
        345678901233333345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678
                   ABCDE

EU      2        2               2                                                         2                       2         2
        0        1               2                                                         3                       4         5
        12345678901 234     567890 1  2345678                                             90         12   345678  901234567890
2F8-G1  NVNHKPSNTKV-DKR-----VEPKSC-D--KTHTCPP---------------------------------------------CP---------AP---ELLGGP--SVFLFPPKPKDT
2F8-G3  NVNHKPSNTKV-DKR-----VELKTP-LGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCP---------AP---ELLGGP--SVFLFPPKPKDT
2F8-G4  NVDHKPSNTKV-DKR-----VESKYG------PPCPS---------------------------------------------CP---------AP---EFLGGP--SVFLFPPKPKDT
KABAT   22          2            2         2                                                                  2           2
        01          2            3         4                                                                  5           6
        9012345678990123333456789012345678901111111111111111111111111111111111111111111111233333333345555678901112345678901 23
                   A    ABC                  ABCDEFGHIJKLMNOPQRSTUVWXYZABCDEFGHIJKLMNOPQRS  ABCDEFGHI  ABC      AB
                                                                       ABCDEFGHIJKLMNOPQRS

EU      2        2         2           2           2           3
        5        6         7           8           9           0
        12345678901234567890123456789  01  2345678901234567  890
2F8-G1  LMISRTPEVTCVVVDVSHEDPEVKFNWYV--DG--VEVHNAKTKPREEQYN--STY
2F8-G4  LMISRTPEVTCVVVDVSQEDPEVQFNWYV--DG--VEVHNAKTKPREEQFN--STY
KABAT   2     2         2         2         3         3
        6     7         8         9         0         1
        45678901234567890123456789012345678901234567890123456789

EU      3        3         3         3           3          3           3         3           3            3         4
        0        1         2         3           4          5           6         7           8            9         0
        12345678901234567890123456789012 3456 78901 2345678901234567  8901234567890123456789  01234 56  78901234567890
2F8-G1  RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI-EKTI-SKAKG-QPREPQVYTLPPSRDE--LTKNQVSLTCLVKGFYPSDIAV--EWESN-GQ--PENNYKTTPPVLDS
2F8-G4  RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI-EKTI-SKAKG-QPREPQVYTLPPSQEE--MTKNQVSLTCLVKGFYPSDIAV--EWESN-GQ--PENNYKTTPPVLDS
KABAT   3         3         3         3          3         3         3         3         4         4         4
        2         3         4         5          6         7         8         9         0         1         2
        01234567890123456789012345678901123456789012345678901234567890123456789012345678901234567890123456789012345678
                                        A
EU       4          4
         0          1         2         3         4
         1  2345678901234567890123456789012345678901234567
2F8-G1  -D--GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
2F8-G4  -D--GSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
KABAT    4         4         4         4         4
         3         4         5         6         7
        90123456789012345678901234567890123456789012345678
```

Figure 17

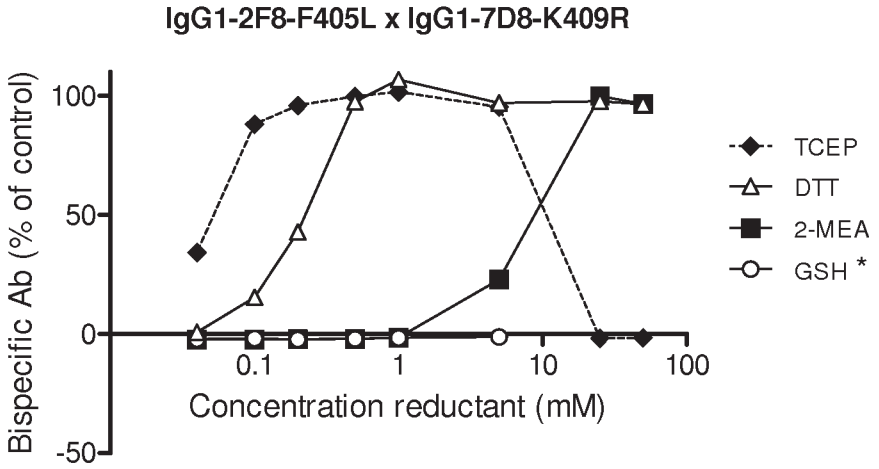
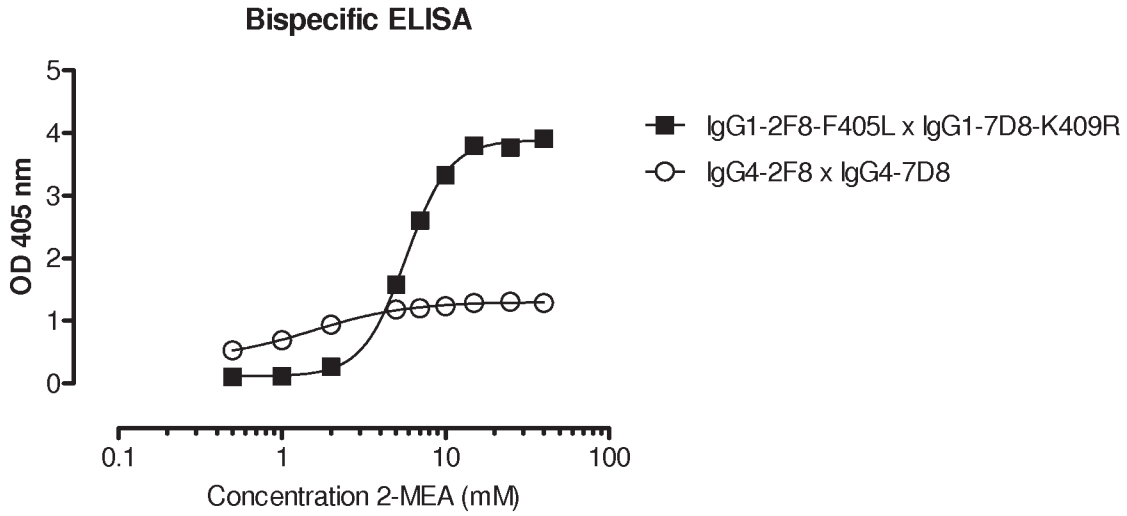

IgG1-2F8-F405L x IgG1-7D8-K409R
Bispecific Ab (% of control)
100
50
0
-50
0.1
1
10
100
Concentration reductant (mM)
TCEP
DTT
2-MEA
GSH *

Figure 18

A


Bispecific ELISA
OD 405 nm
0
1
2
3
4
5
0.1
1
10
100
Concentration 2-MEA (mM)
IgG1-2F8-F405L x IgG1-7D8-K409R
IgG4-2F8 x IgG4-7D8

B

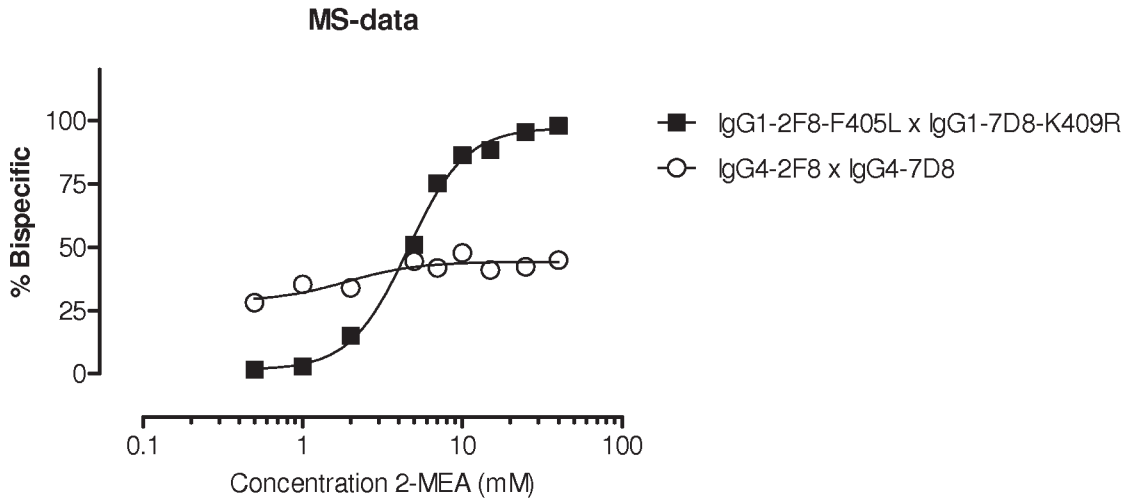
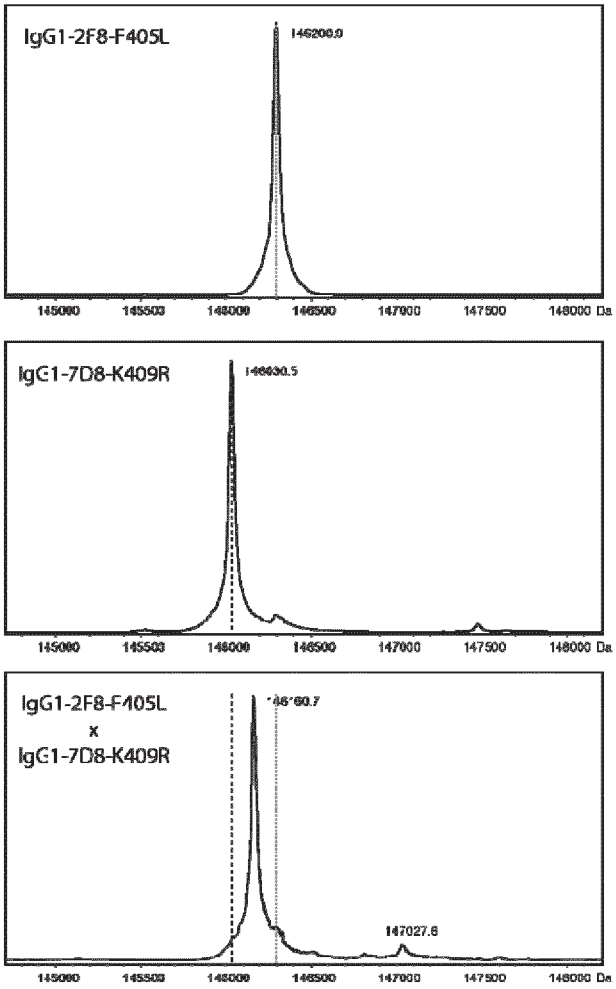

MS-data
% Bispecific
0
25
50
75
100
0.1
1
10
100
Concentration 2-MEA (mM)
IgG1-2F8-F405L x IgG1-7D8-K409R
IgG4-2F8 x IgG4-7D8

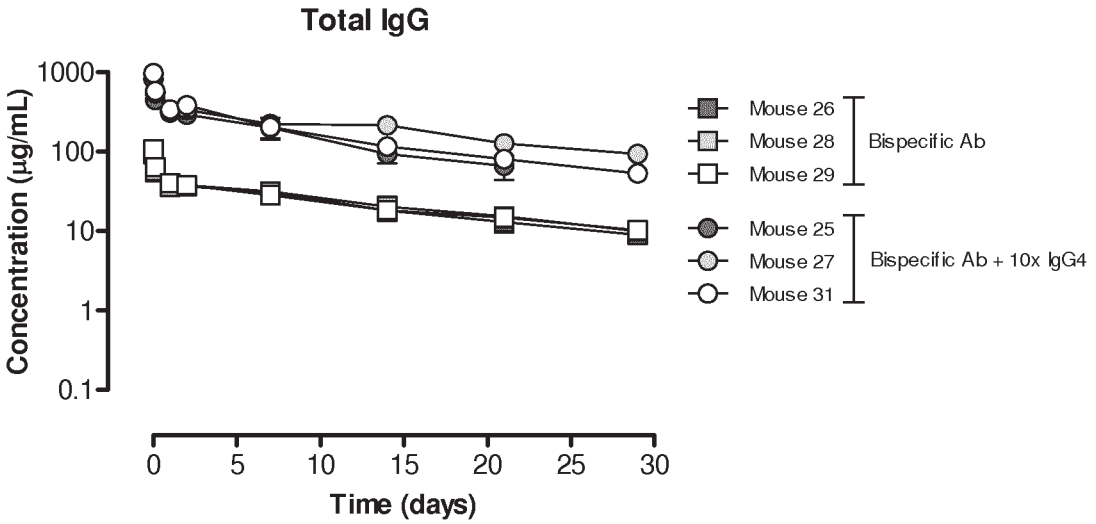
Figure 19



	Measured
IgG1-2F8-F405L	146,290.9
IgG1-7D8-K409R	146,030.5
IgG1-2F8-F405L x IgG1-7D8-	146,160.7

Figure 20

A


Total IgG
Concentration (µg/mL)
1000
100
10
1
0.1
0
5
10
15
20
25
30
Time (days)
Mouse 26
Mouse 28
Mouse 29
Bispecific Ab
Mouse 25
Mouse 27
Mouse 31
Bispecific Ab + 10x IgG4

B

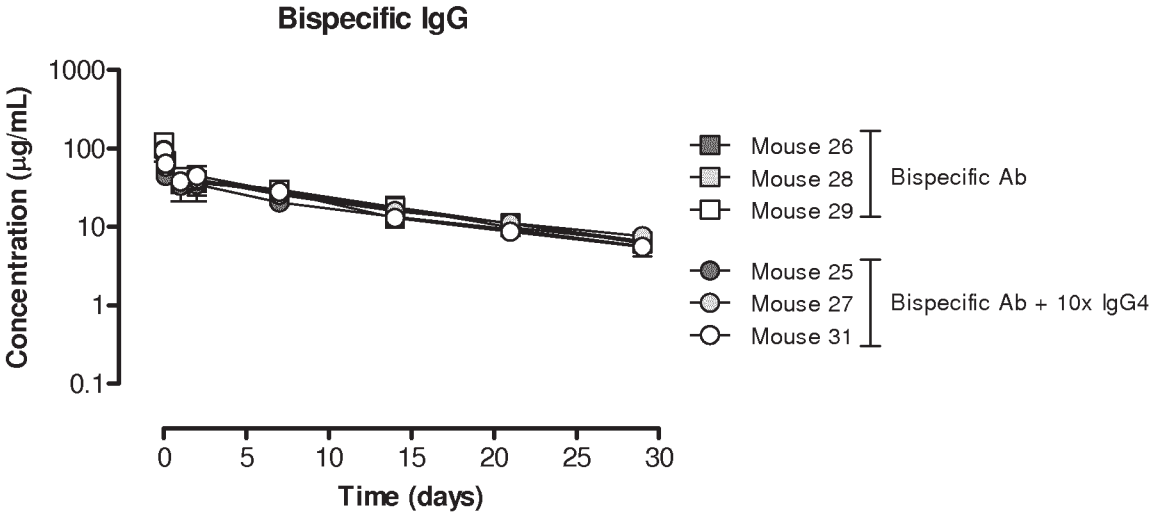
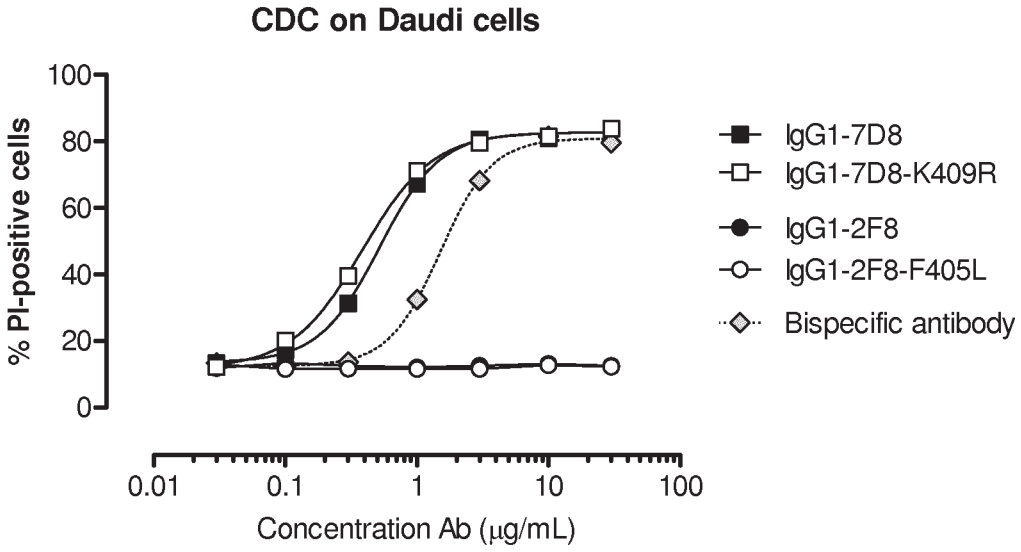

Bispecific IgG
Concentration (µg/mL)
1000
100
10
1
0.1
0
5
10
15
20
25
30
Time (days)
Mouse 26
Mouse 28
Mouse 29
Bispecific Ab
Mouse 25
Mouse 27
Mouse 31
Bispecific Ab + 10x IgG4

Figure 21

A


CDC on Daudi cells
% PI-positive cells
100
80
60
40
20
0
0.01
0.1
1
10
100
Concentration Ab (µg/mL)
IgG1-7D8
IgG1-7D8-K409R
IgG1-2F8
IgG1-2F8-F405L
Bispecific antibody

B

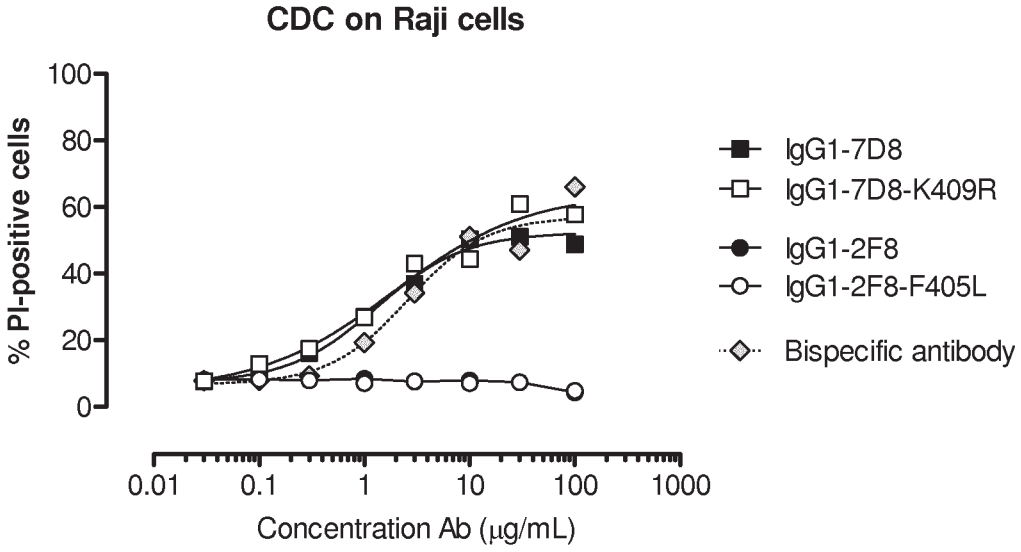

CDC on Raji cells
% PI-positive cells
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration Ab (µg/mL)
IgG1-7D8
IgG1-7D8-K409R
IgG1-2F8
IgG1-2F8-F405L
Bispecific antibody

Figure 22

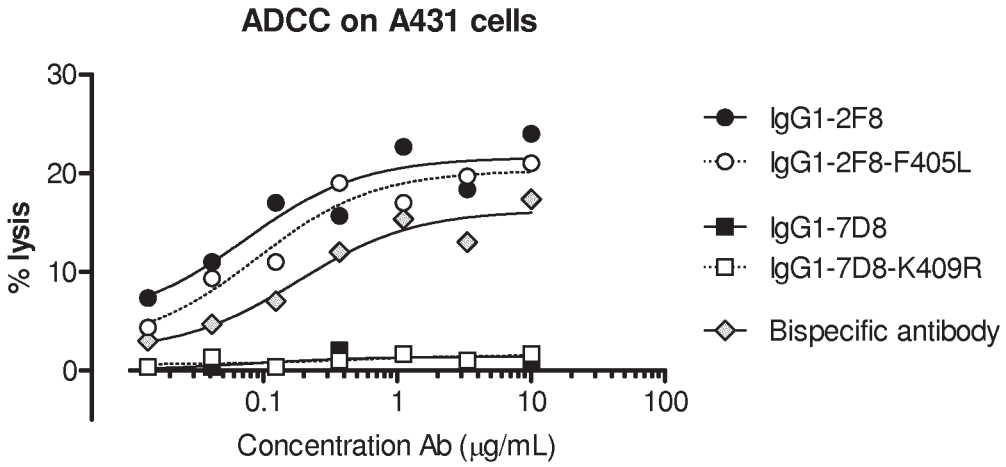
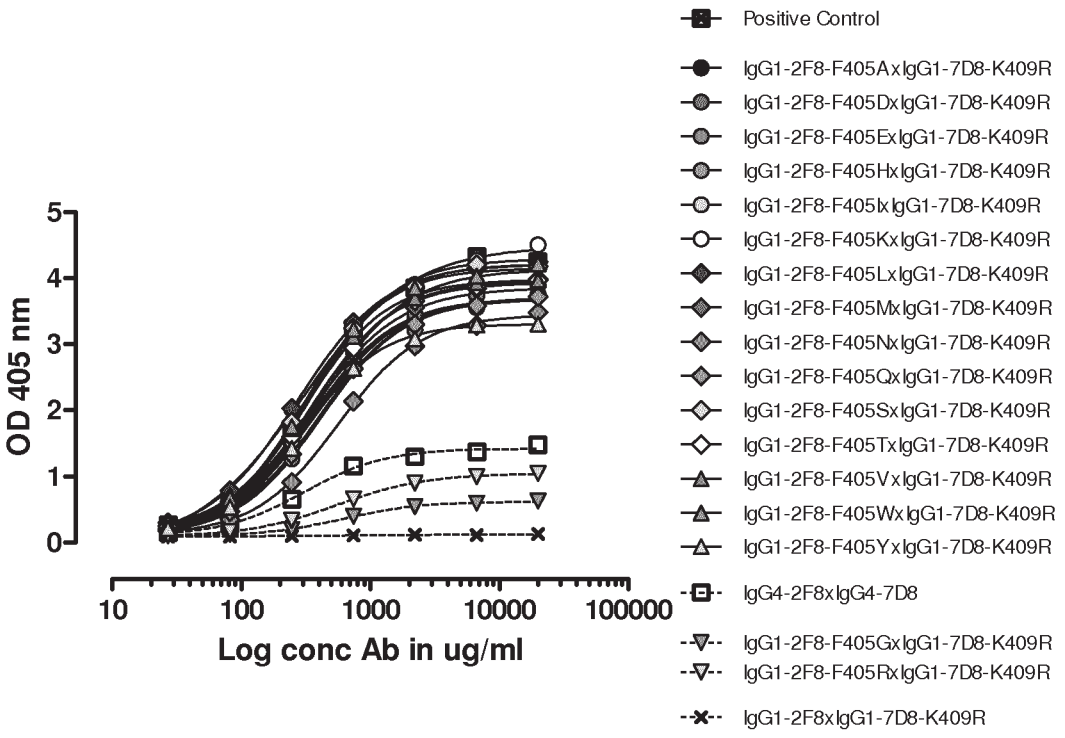
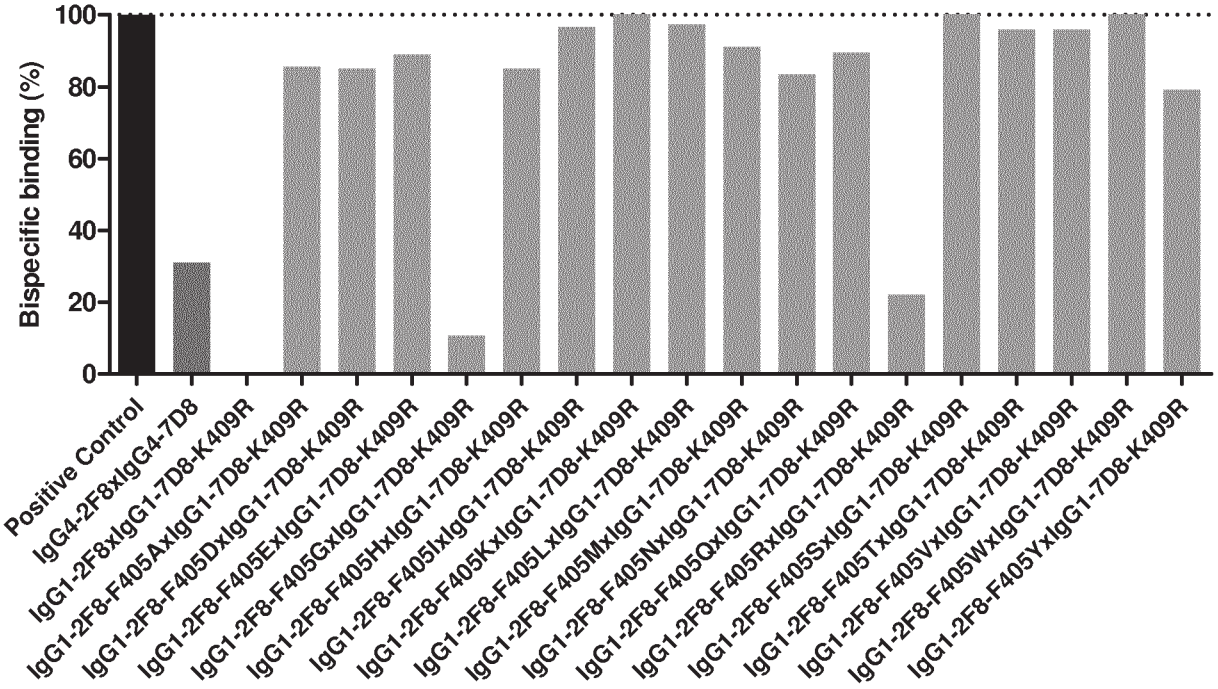

ADCC on A431 cells
% lysis
30
20
10
0
0.1
1
10
100
Concentration Ab (µg/mL)
IgG1-2F8
IgG1-2F8-F405L
IgG1-7D8
IgG1-7D8-K409R
Bispecific antibody

Figure 23

A



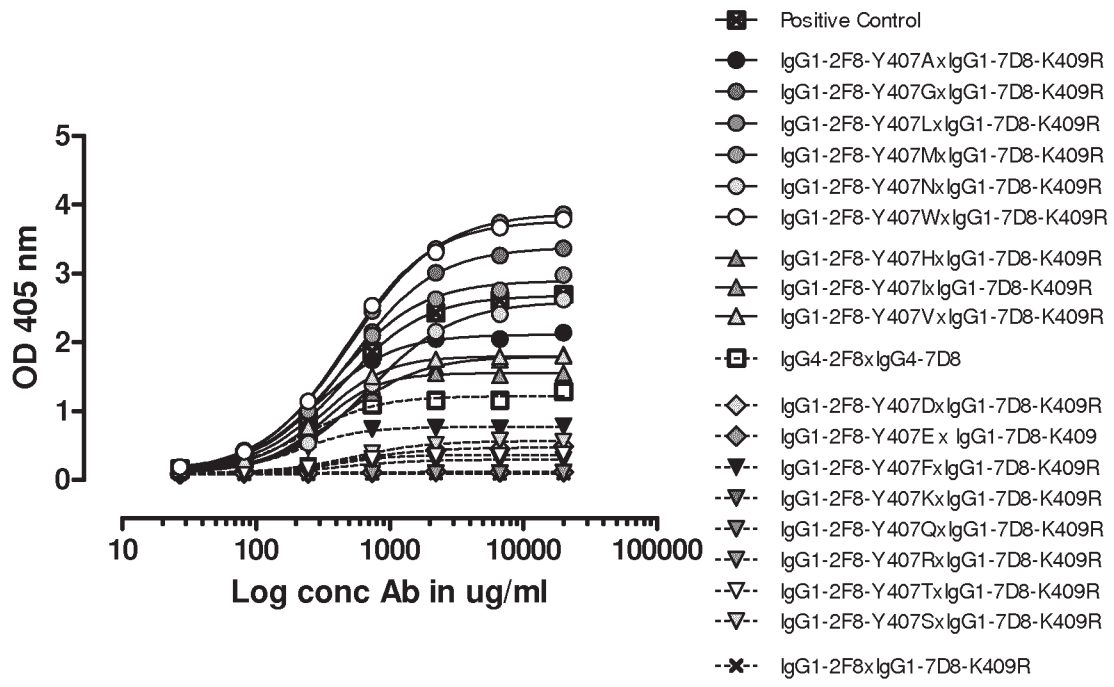
B



B

Figure 24

A


OD 405 nm
0
1
2
3
4
5
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG1-2F8-Y407AxIgG1-7D8-K409R
IgG1-2F8-Y407GxIgG1-7D8-K409R
IgG1-2F8-Y407LxIgG1-7D8-K409R
IgG1-2F8-Y407MxIgG1-7D8-K409R
IgG1-2F8-Y407NxIgG1-7D8-K409R
IgG1-2F8-Y407WxIgG1-7D8-K409R
IgG1-2F8-Y407HxIgG1-7D8-K409R
IgG1-2F8-Y407IxIgG1-7D8-K409R
IgG1-2F8-Y407VxIgG1-7D8-K409R
IgG4-2F8xIgG4-7D8
IgG1-2F8-Y407DxIgG1-7D8-K409R
IgG1-2F8-Y407E x IgG1-7D8-K409
IgG1-2F8-Y407FxIgG1-7D8-K409R
IgG1-2F8-Y407KxIgG1-7D8-K409R
IgG1-2F8-Y407QxIgG1-7D8-K409R
IgG1-2F8-Y407RxIgG1-7D8-K409R
IgG1-2F8-Y407TxIgG1-7D8-K409R
IgG1-2F8-Y407SxIgG1-7D8-K409R
IgG1-2F8xIgG1-7D8-K409R

B

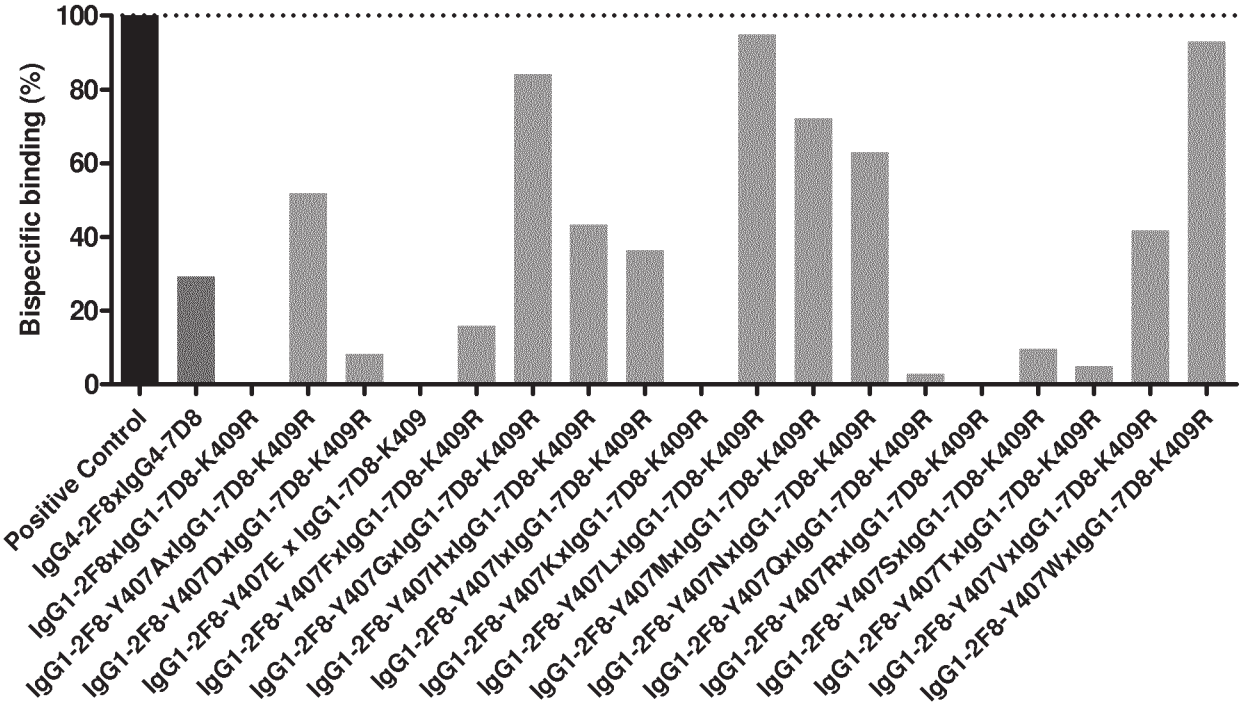

Bispecific binding (%)
0
20
40
60
80
100
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-Y407AxIgG1-7D8-K409R
IgG1-2F8-Y407DxIgG1-7D8-K409R
IgG1-2F8-Y407E x IgG1-7D8-K409
IgG1-2F8-Y407FxIgG1-7D8-K409R
IgG1-2F8-Y407GxIgG1-7D8-K409R
IgG1-2F8-Y407HxIgG1-7D8-K409R
IgG1-2F8-Y407IxIgG1-7D8-K409R
IgG1-2F8-Y407KxIgG1-7D8-K409R
IgG1-2F8-Y407LxIgG1-7D8-K409R
IgG1-2F8-Y407MxIgG1-7D8-K409R
IgG1-2F8-Y407NxIgG1-7D8-K409R
IgG1-2F8-Y407QxIgG1-7D8-K409R
IgG1-2F8-Y407RxIgG1-7D8-K409R
IgG1-2F8-Y407SxIgG1-7D8-K409R
IgG1-2F8-Y407TxIgG1-7D8-K409R
IgG1-2F8-Y407VxIgG1-7D8-K409R
IgG1-2F8-Y407WxIgG1-7D8-K409R

Figure 25

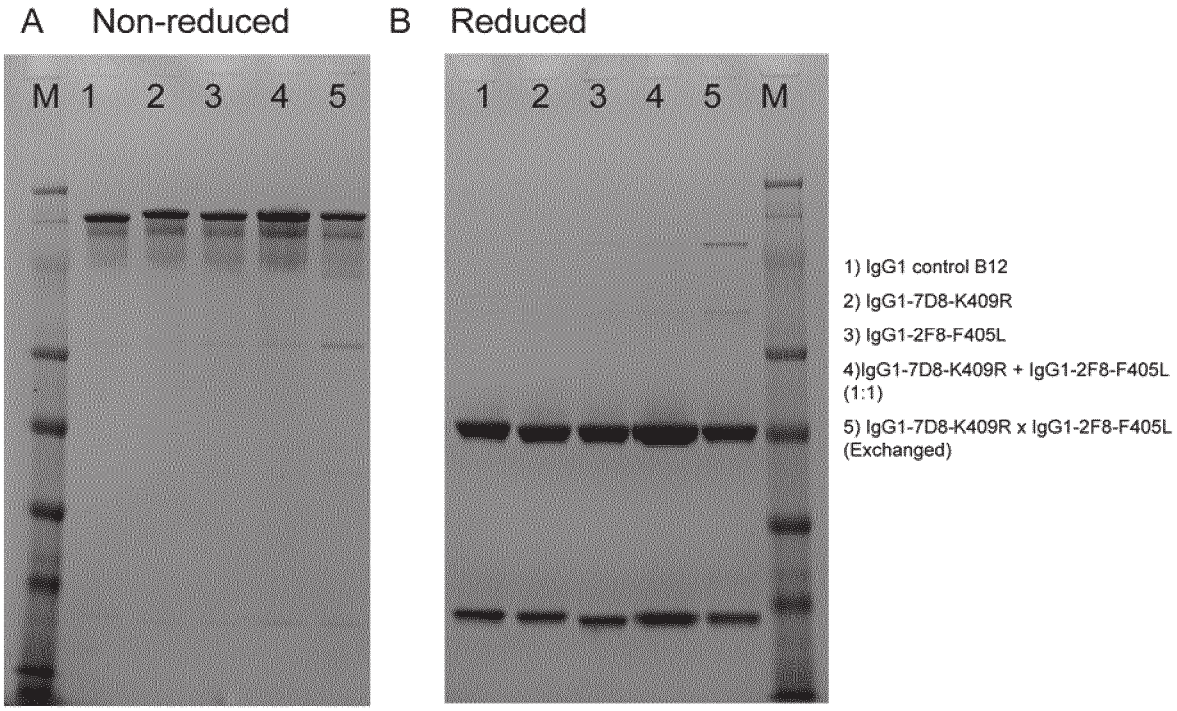

A Non-reduced
M 1 2 3 4 5
B Reduced
1 2 3 4 5 M
1) IgG1 control B12
2) IgG1-7D8-K409R
3) IgG1-2F8-F405L
4)IgG1-7D8-K409R + IgG1-2F8-F405L (1:1)
5) IgG1-7D8-K409R x IgG1-2F8-F405L (Exchanged)

Figure 26

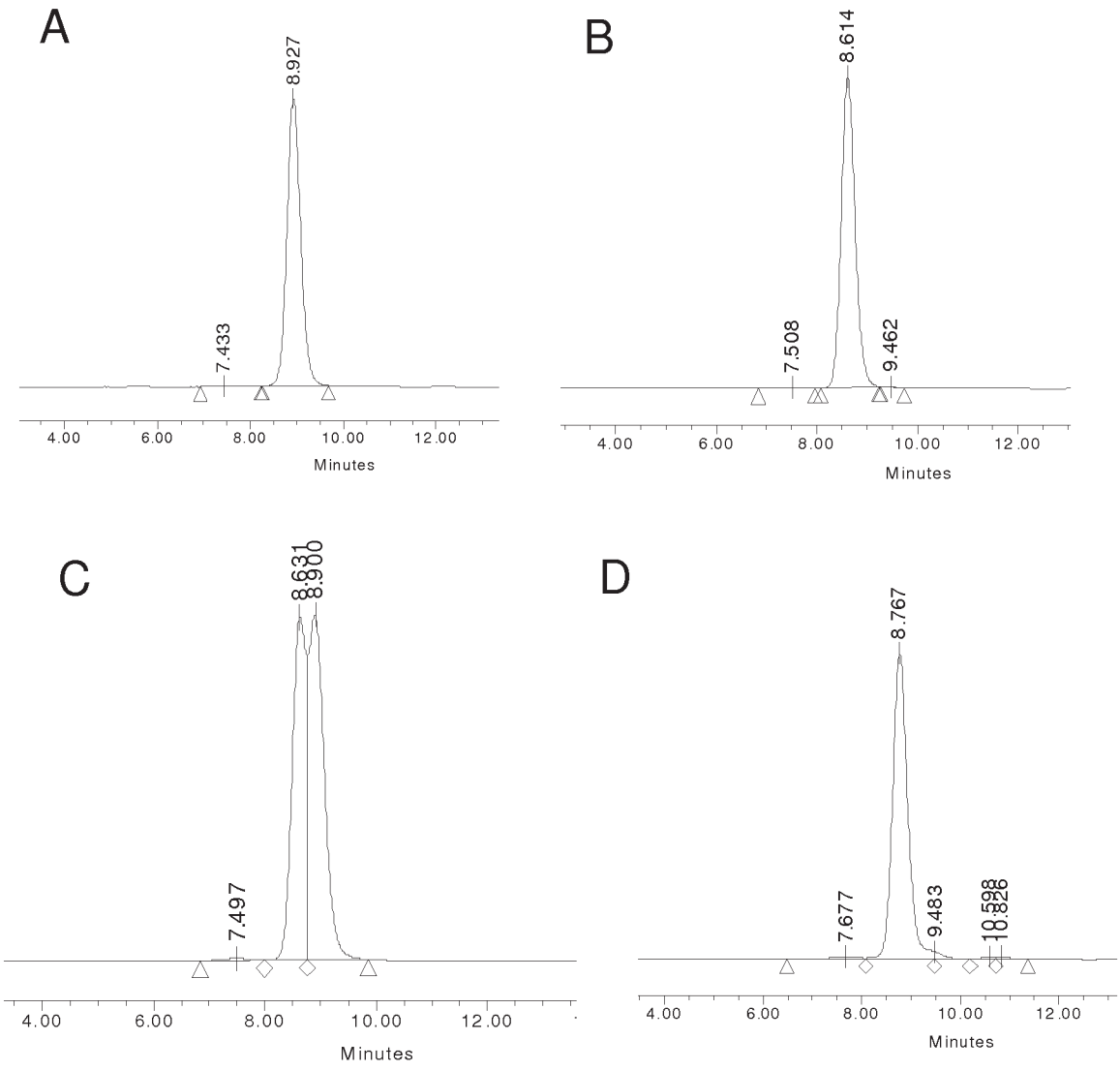

A
8.927
7.433
4.00
6.00
8.00
10.00
12.00
Minutes
B
8.614
7.508
9.462
4.00
6.00
8.00
10.00
12.00
Minutes
C
8.631
8.900
7.497
4.00
6.00
8.00
10.00
12.00
Minutes
D
8.767
7.677
9.483
10.528
10.828
4.00
6.00
8.00
10.00
12.00
Minutes

Figure 27

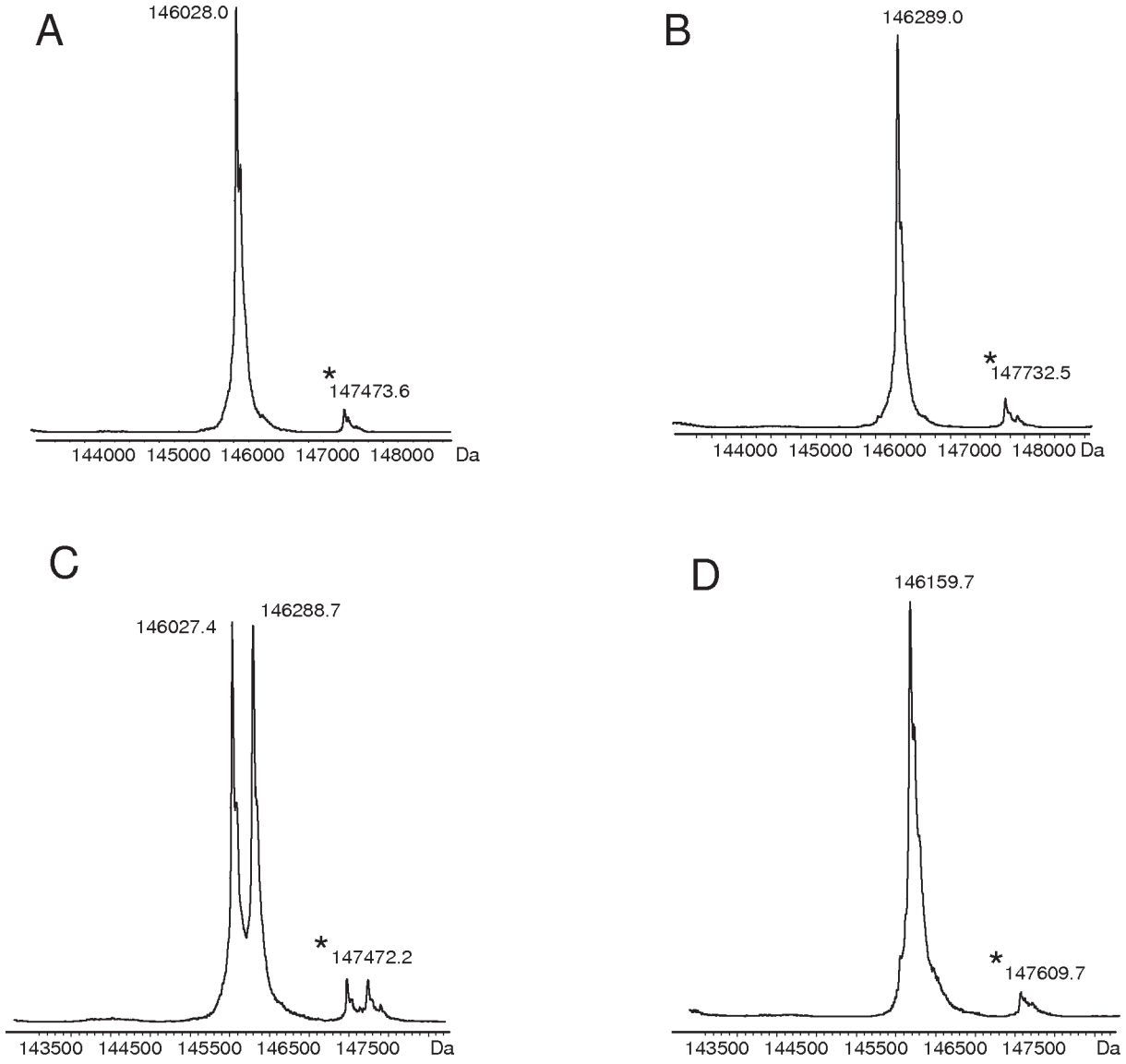

A
146028.0
*
147473.6
144000 145000 146000 147000 148000 Da
B
146289.0
*
147732.5
144000 145000 146000 147000 148000 Da
C
146027.4
146288.7
*
147472.2
143500 144500 145500 146500 147500 Da
D
146159.7
*
147609.7
143500 144500 145500 146500 147500 Da

Figure 28

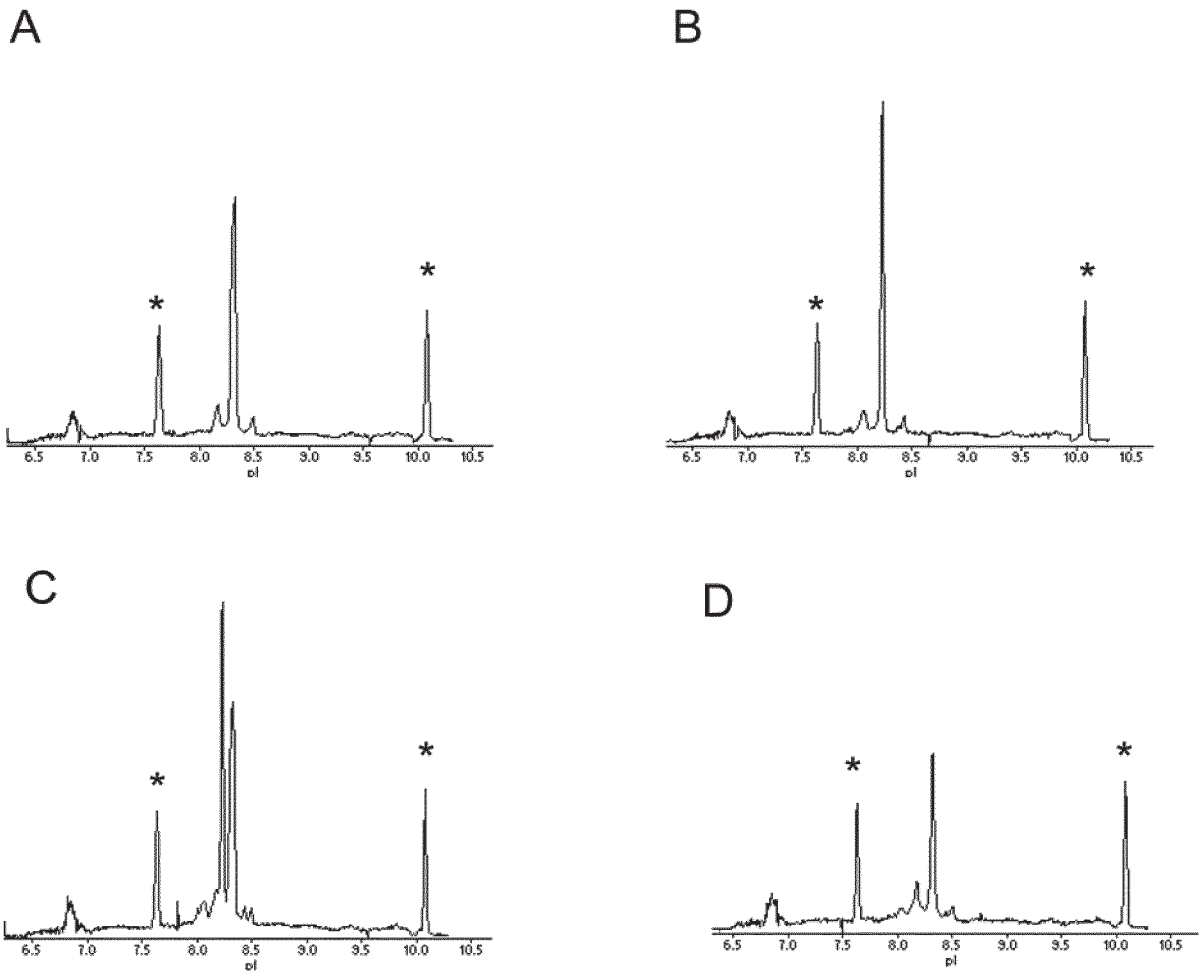


Figure 29

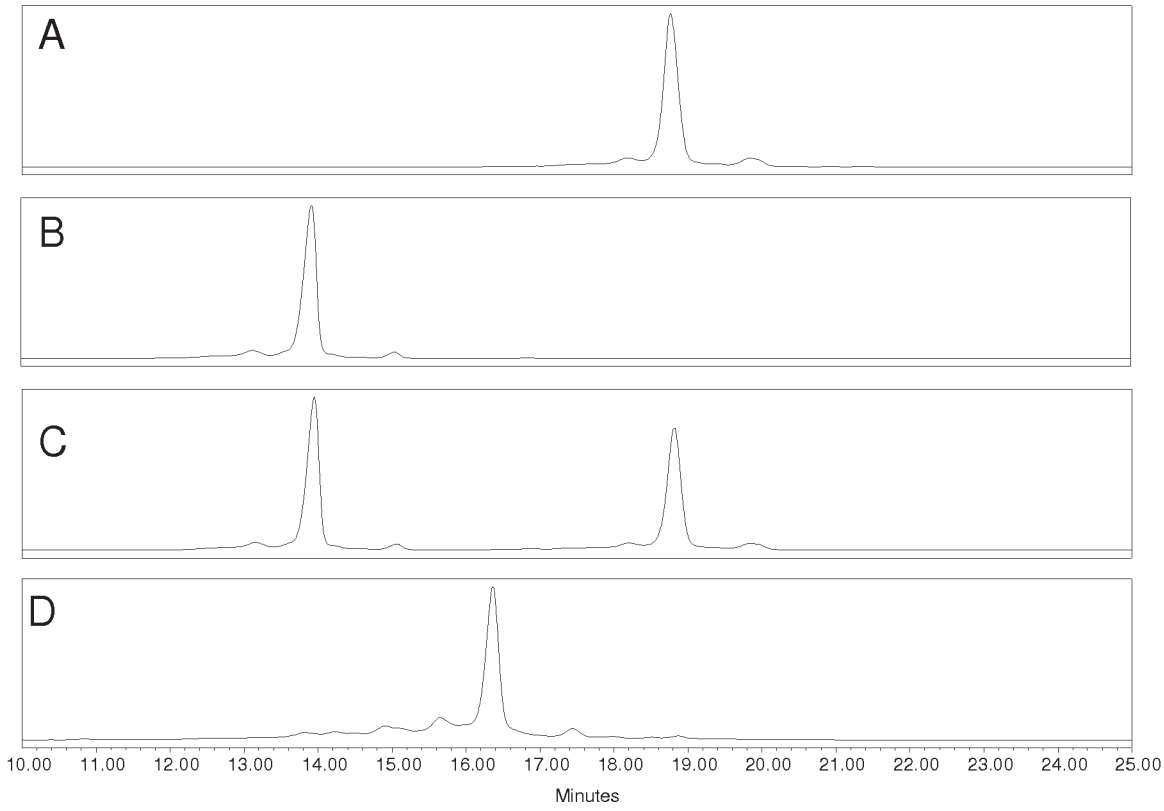

A
B
C
D
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
22.00
23.00
24.00
25.00
Minutes

Figure 30

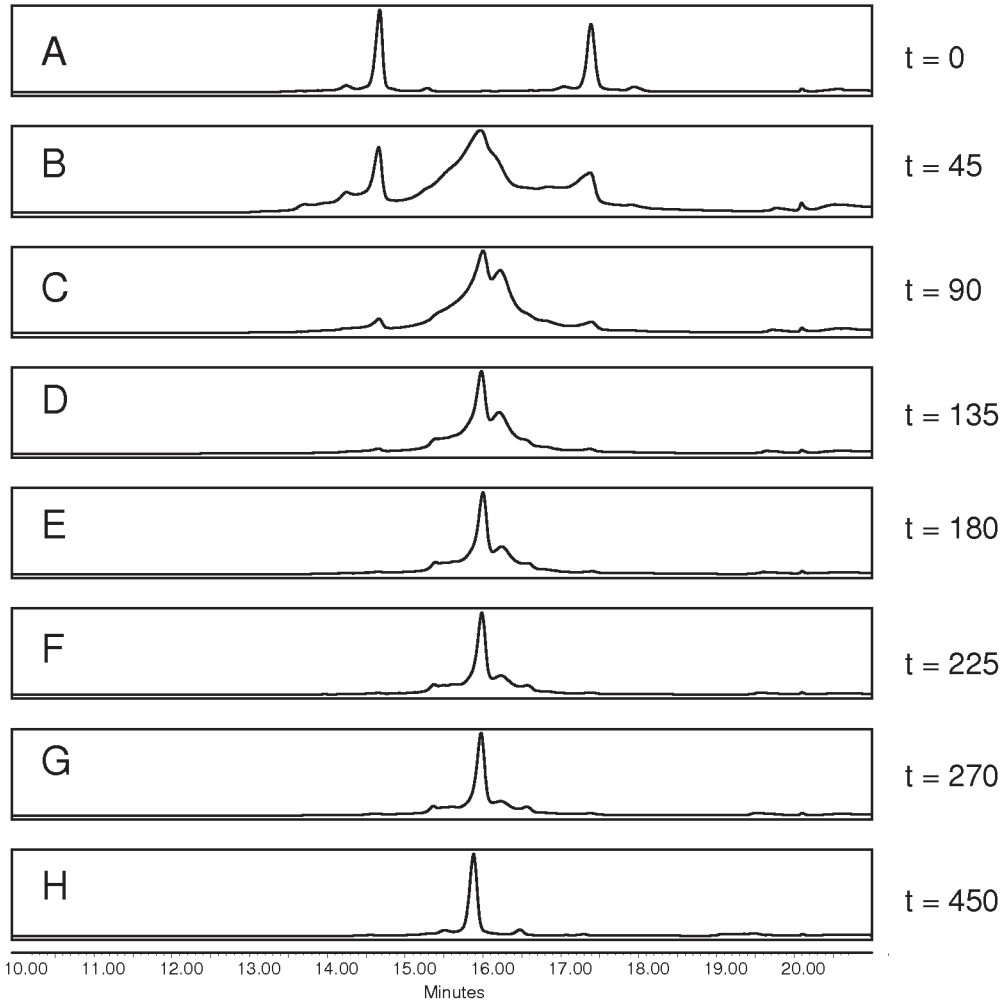

A
B
C
D
E
F
G
H
t = 0
t = 45
t = 90
t = 135
t = 180
t = 225
t = 270
t = 450
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
Minutes

Figure 31

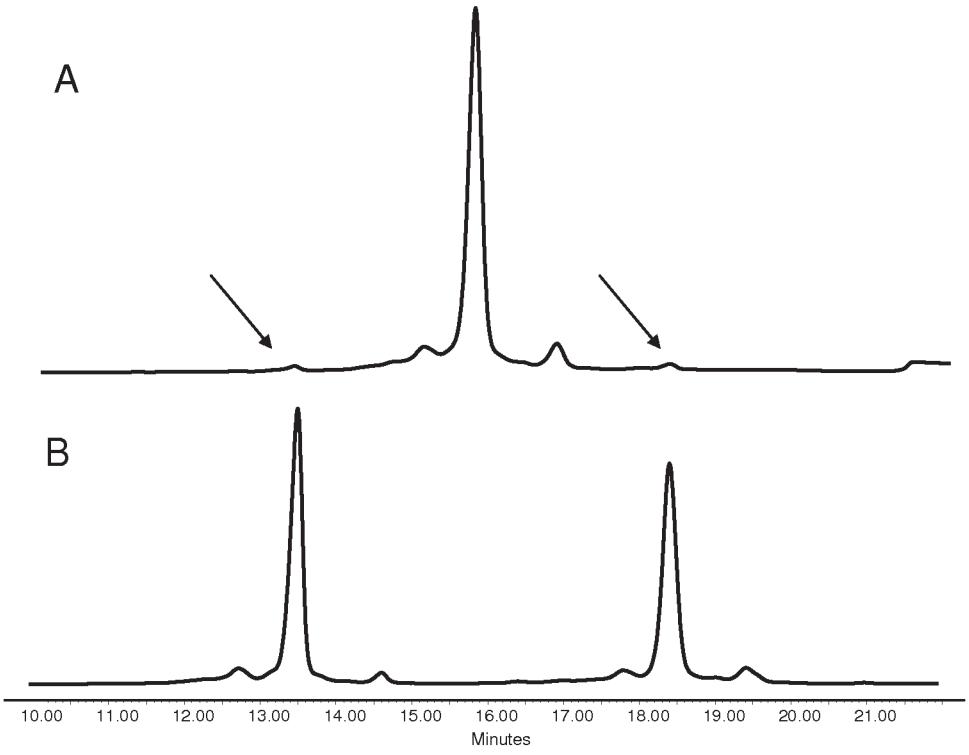
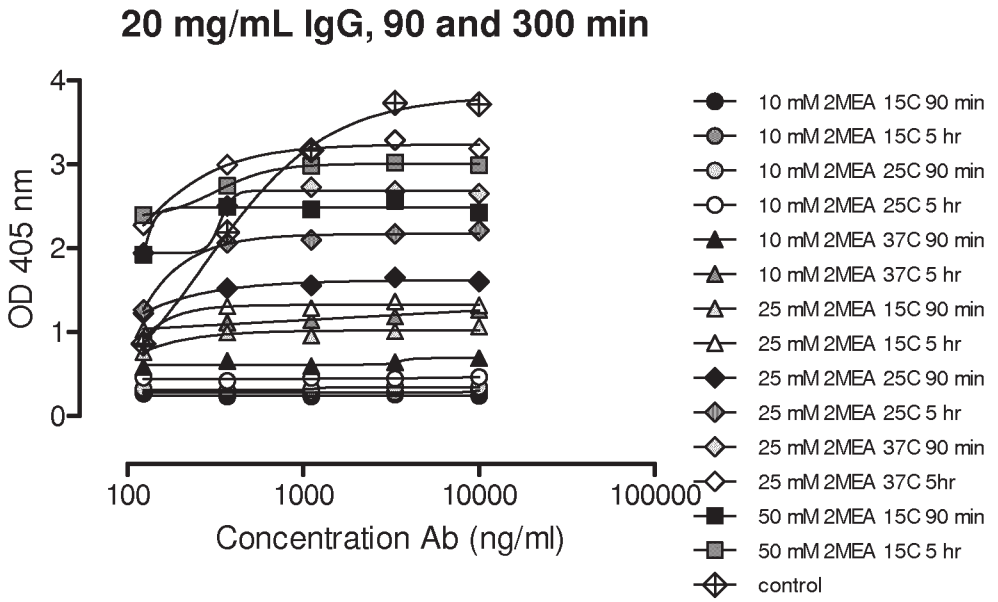

A
B
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
Minutes

Figure 32

A


20 mg/mL IgG, 90 and 300 min
OD 405 nm
0
1
2
3
4
100
1000
10000
100000
Concentration Ab (ng/ml)
10 mM 2MEA 15C 90 min
10 mM 2MEA 15C 5 hr
10 mM 2MEA 25C 90 min
10 mM 2MEA 25C 5 hr
10 mM 2MEA 37C 90 min
10 mM 2MEA 37C 5 hr
25 mM 2MEA 15C 90 min
25 mM 2MEA 15C 5 hr
25 mM 2MEA 25C 90 min
25 mM 2MEA 25C 5 hr
25 mM 2MEA 37C 90 min
25 mM 2MEA 37C 5hr
50 mM 2MEA 15C 90 min
50 mM 2MEA 15C 5 hr
control

B

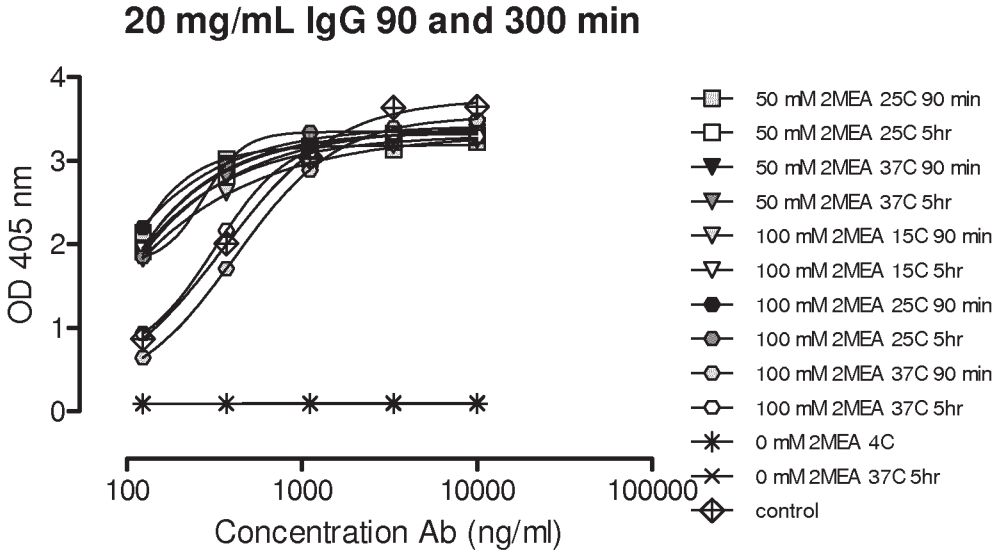
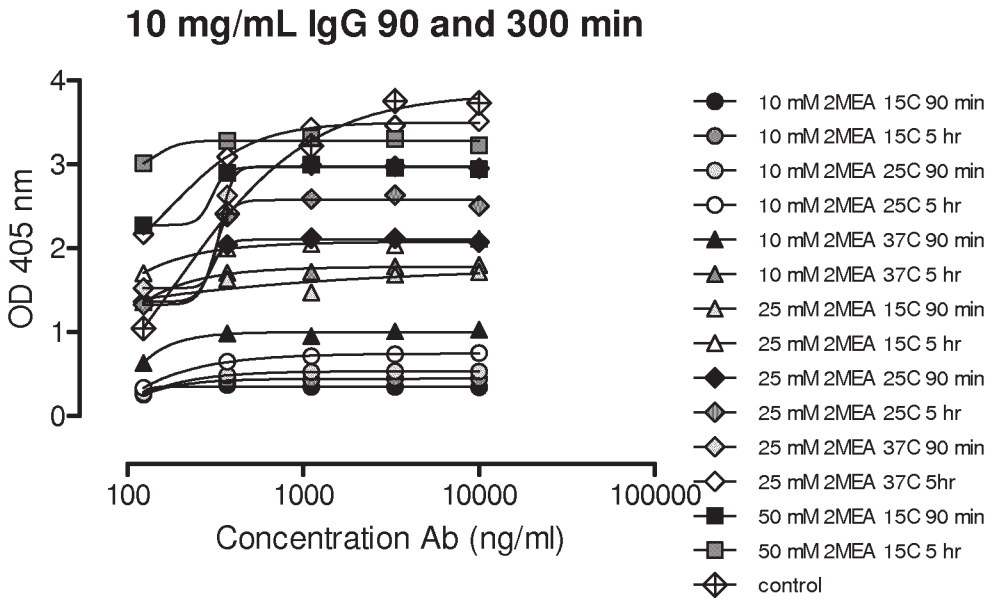

20 mg/mL IgG 90 and 300 min
OD 405 nm
0
1
2
3
4
100
1000
10000
100000
Concentration Ab (ng/ml)
50 mM 2MEA 25C 90 min
50 mM 2MEA 25C 5hr
50 mM 2MEA 37C 90 min
50 mM 2MEA 37C 5hr
100 mM 2MEA 15C 90 min
100 mM 2MEA 15C 5hr
100 mM 2MEA 25C 90 min
100 mM 2MEA 25C 5hr
100 mM 2MEA 37C 90 min
100 mM 2MEA 37C 5hr
0 mM 2MEA 4C
0 mM 2MEA 37C 5hr
control

Figure 32 (continued)

C



D

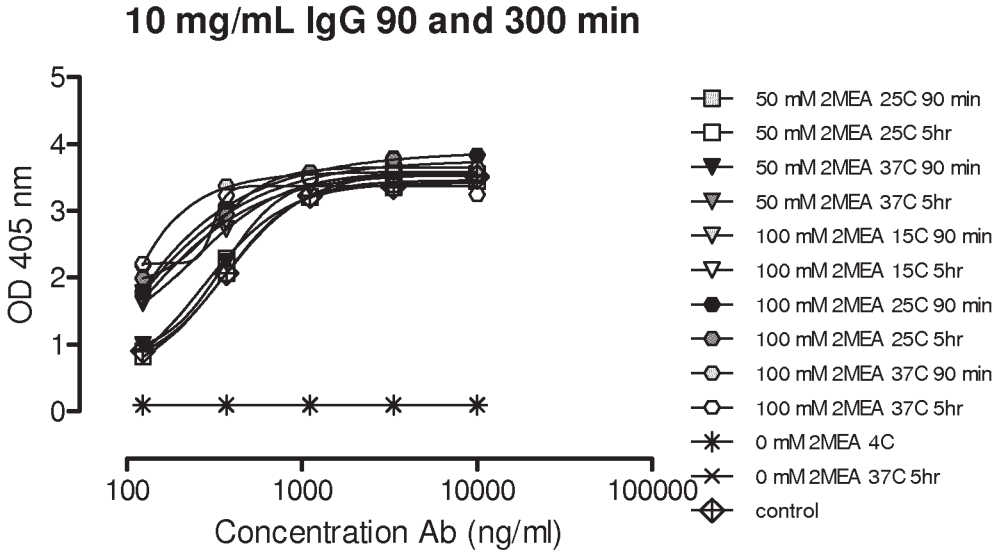
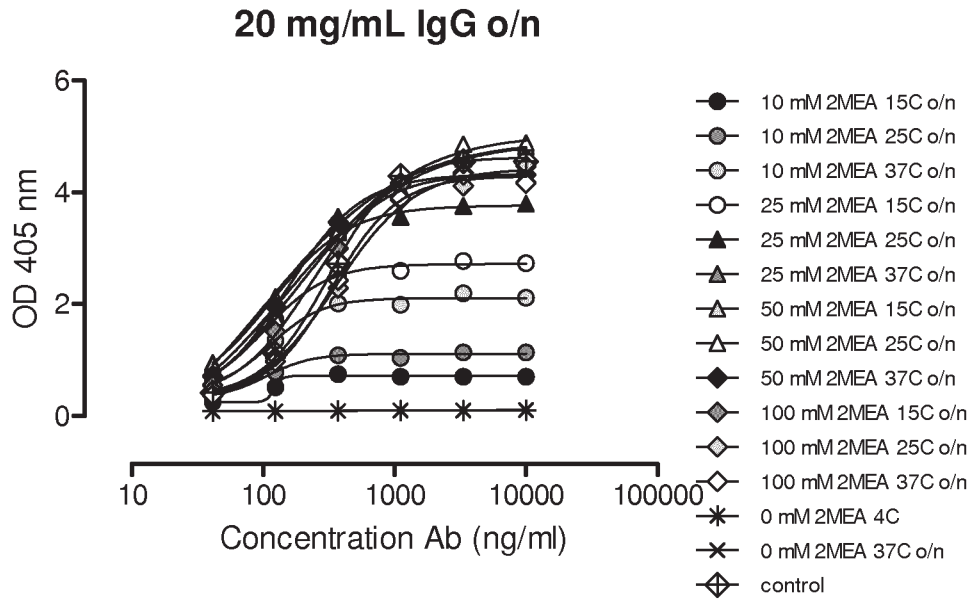


Figure 32 (continued)

E



F

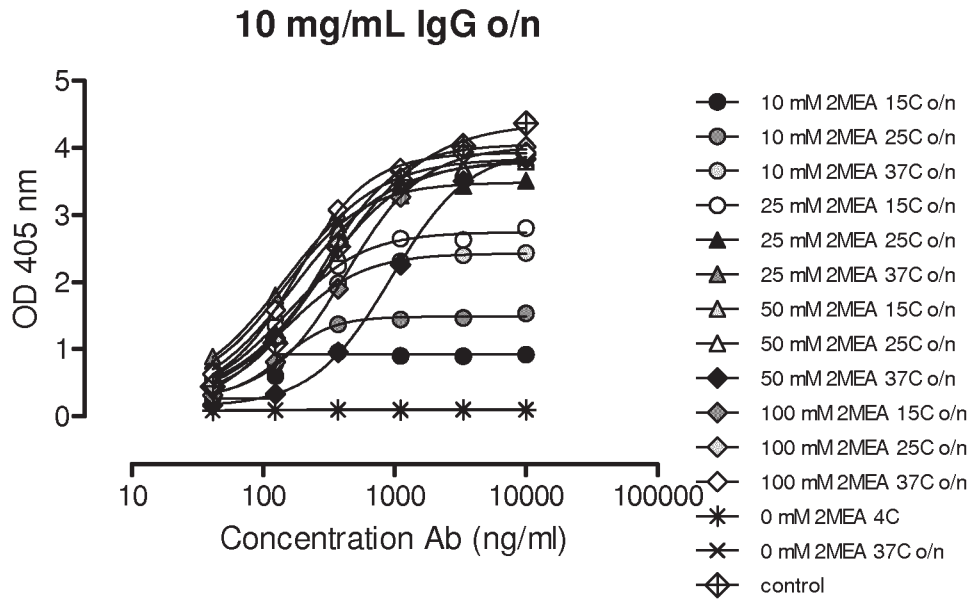
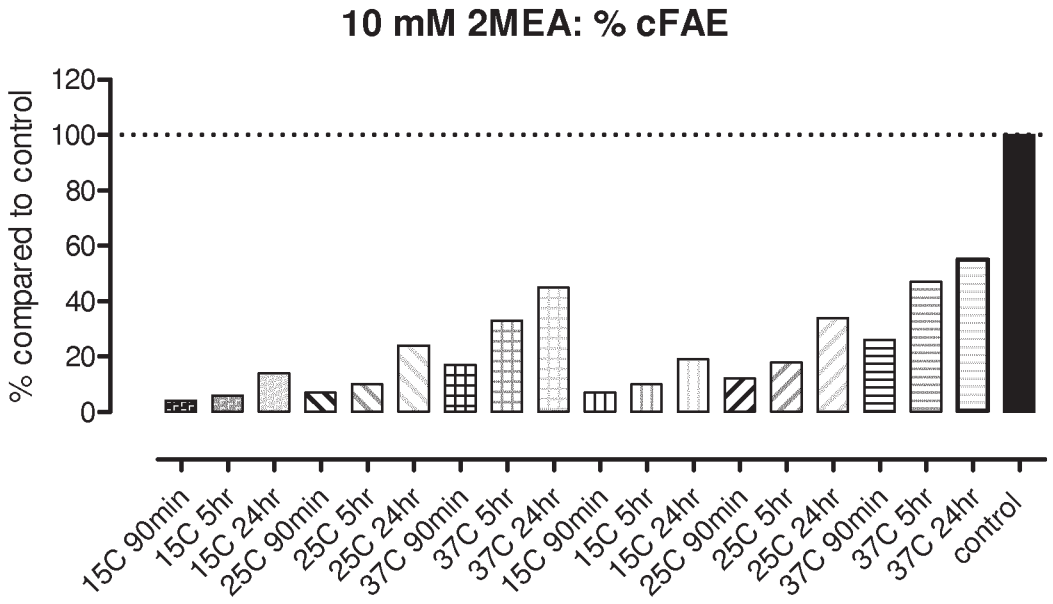


Figure 33

A


10 mM 2MEA: % cFAE
% compared to control
120
100
80
60
40
20
0
15C 90min
15C 5hr
15C 24hr
25C 90min
25C 5hr
25C 24hr
37C 90min
37C 5hr
37C 24hr
15C 90min
15C 5hr
15C 24hr
25C 90min
25C 5hr
25C 24hr
37C 90min
37C 5hr
37C 24hr
control

B

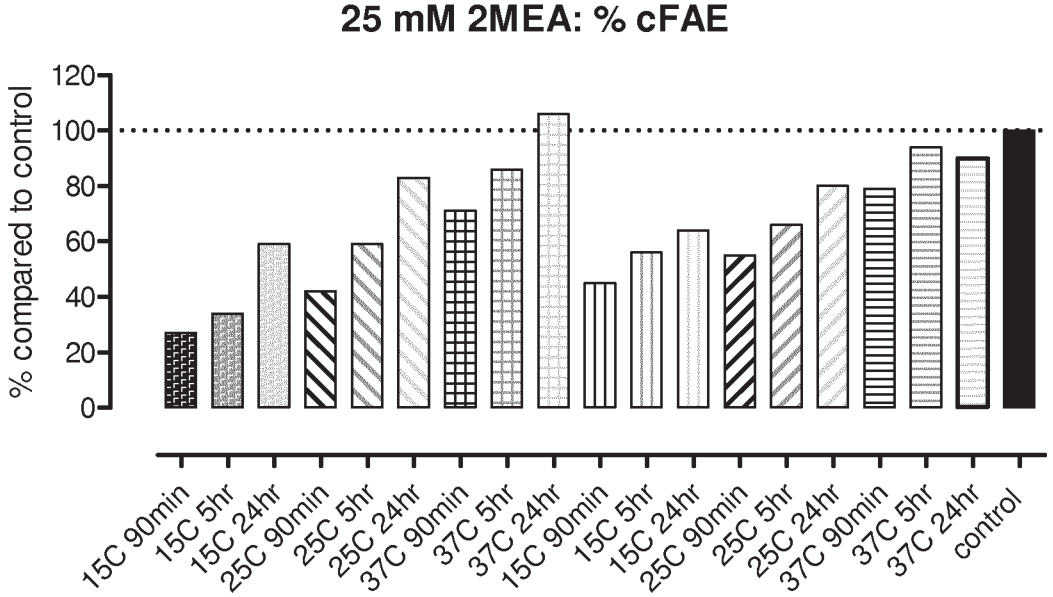
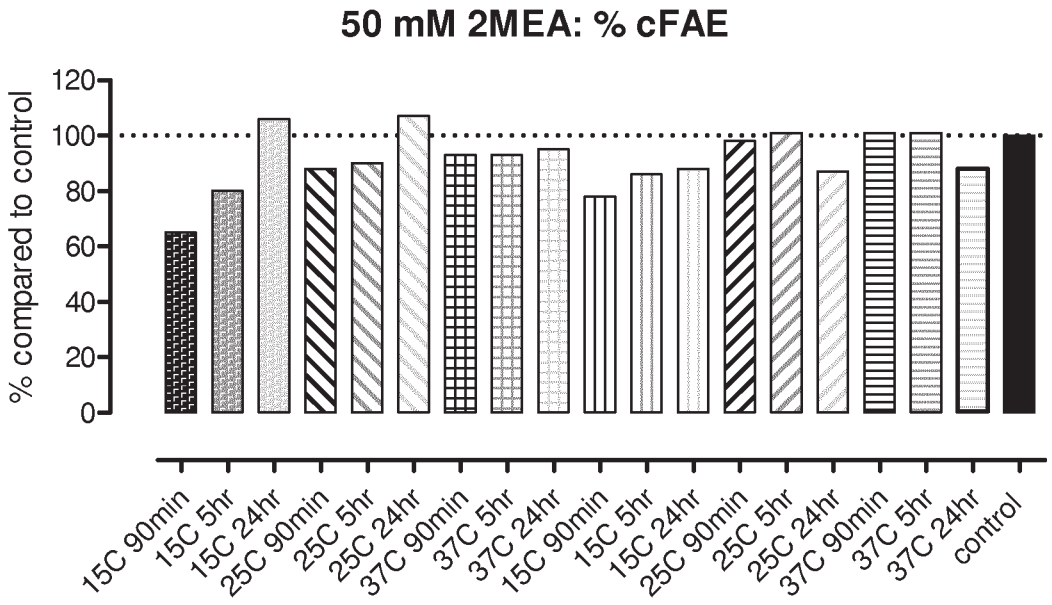

25 mM 2MEA: % cFAE
% compared to control
120
100
80
60
40
20
0
15C 90min
15C 5hr
15C 24hr
25C 90min
25C 5hr
25C 24hr
37C 90min
37C 5hr
37C 24hr
15C 90min
15C 5hr
15C 24hr
25C 90min
25C 5hr
25C 24hr
37C 90min
37C 5hr
37C 24hr
control

Figure 33 (continued)

C



D

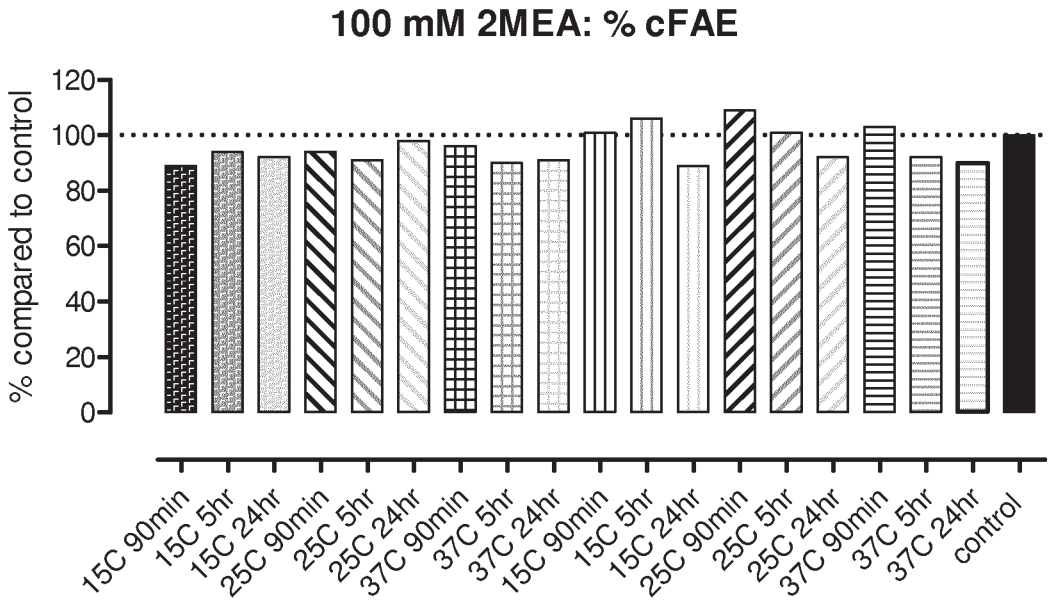


Figure 34

A

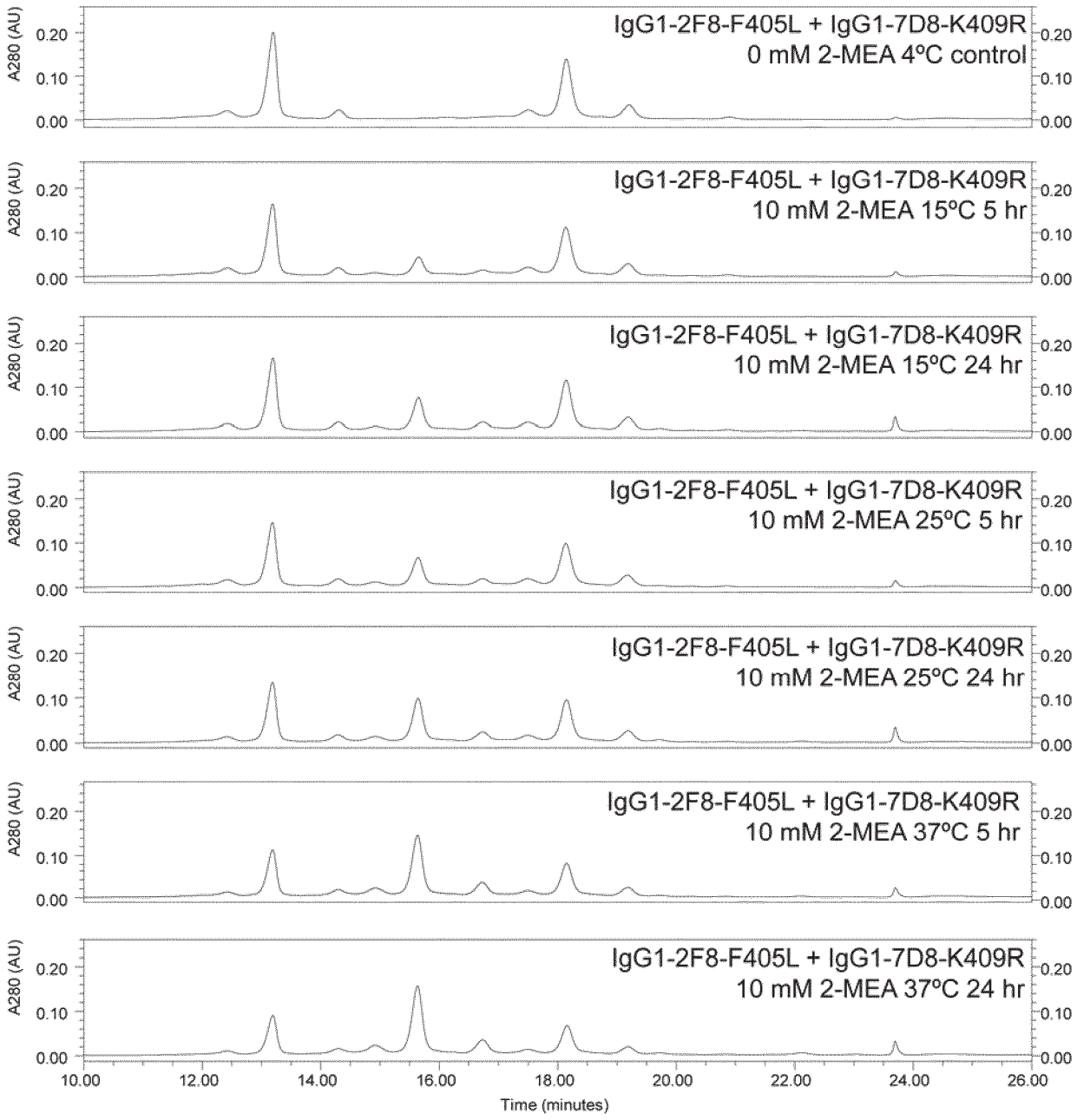

IgG1-2F8-F405L + IgG1-7D8-K409R
0 mM 2-MEA 4ºC control
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 15ºC 5 hr
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 15ºC 24 hr
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 25ºC 5 hr
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 25ºC 24 hr
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 37ºC 5 hr
IgG1-2F8-F405L + IgG1-7D8-K409R
10 mM 2-MEA 37ºC 24 hr
A280 (AU)
0.00
0.10
0.20
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
Time (minutes)

Figure 34 (continued)

B

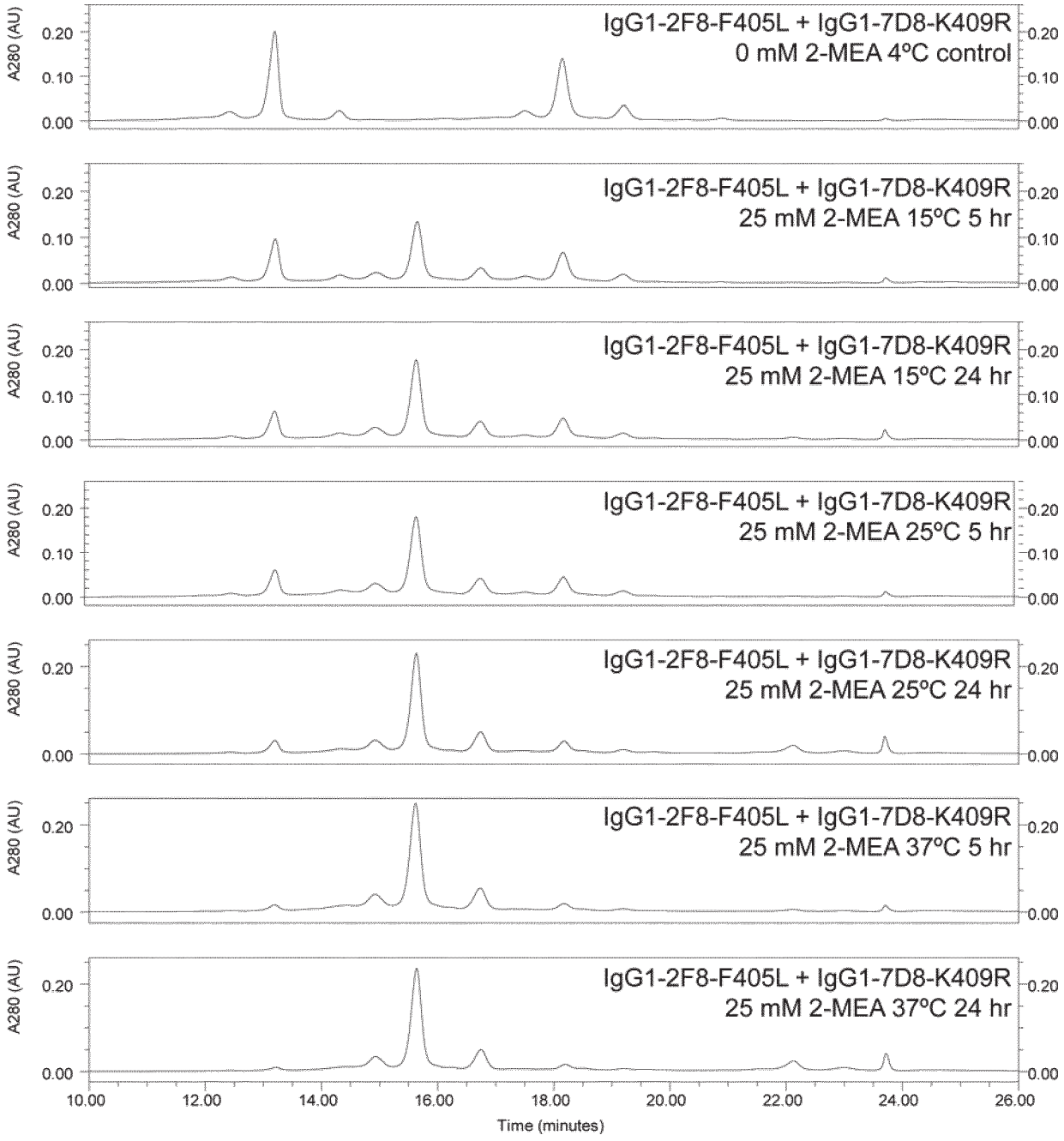


Figure 34 (continued)

C

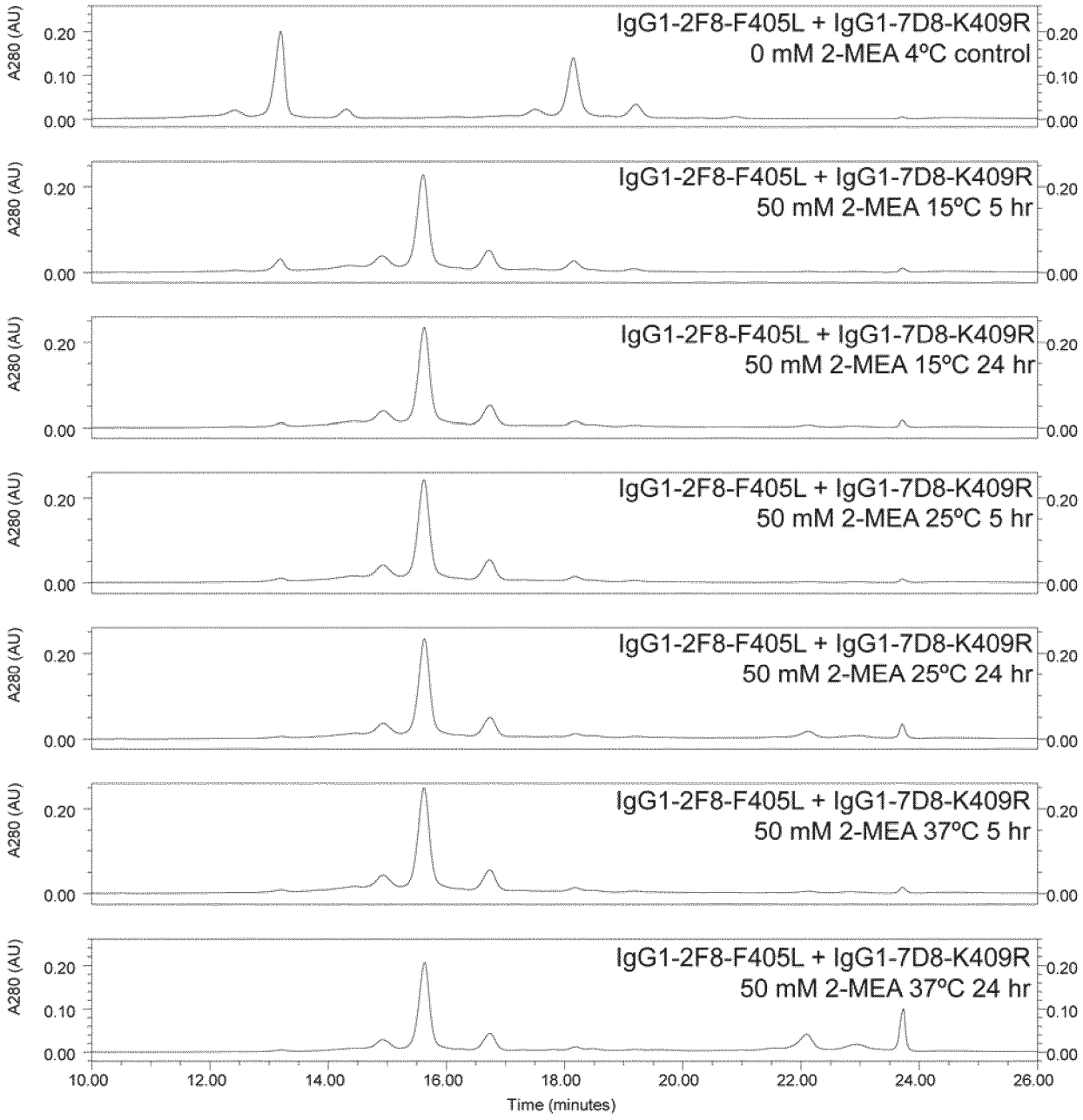


Figure 34 (continued)

D

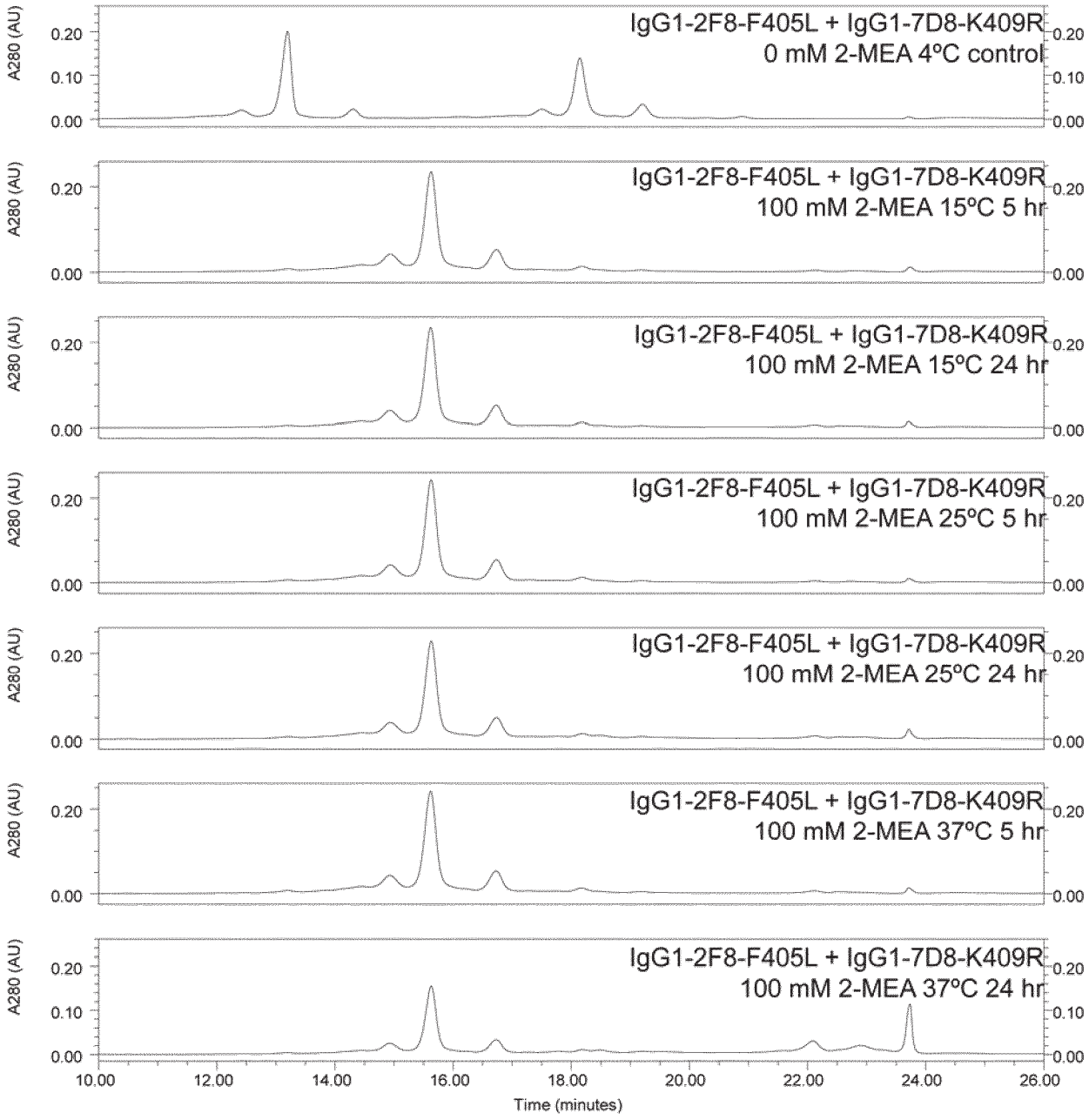
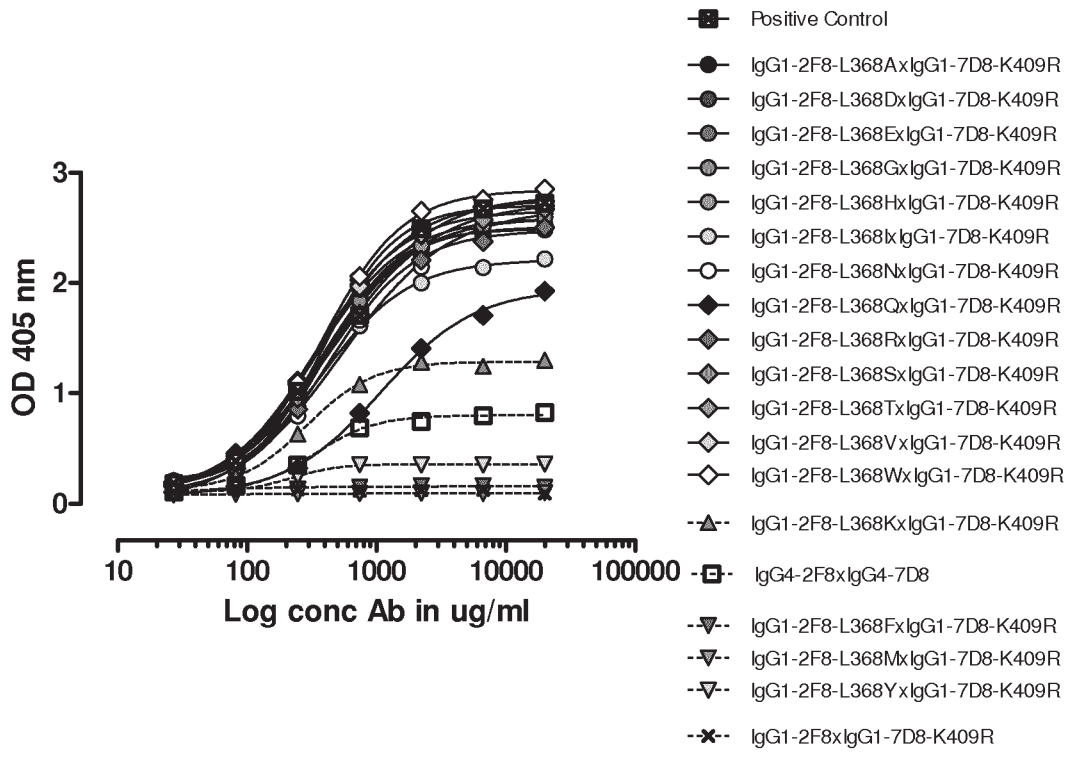


Figure 35

A


OD 405 nm
3
2
1
0
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG1-2F8-L368AxIgG1-7D8-K409R
IgG1-2F8-L368DxIgG1-7D8-K409R
IgG1-2F8-L368ExIgG1-7D8-K409R
IgG1-2F8-L368GxIgG1-7D8-K409R
IgG1-2F8-L368HxIgG1-7D8-K409R
IgG1-2F8-L368IxIgG1-7D8-K409R
IgG1-2F8-L368NxIgG1-7D8-K409R
IgG1-2F8-L368QxIgG1-7D8-K409R
IgG1-2F8-L368RxIgG1-7D8-K409R
IgG1-2F8-L368SxIgG1-7D8-K409R
IgG1-2F8-L368TxIgG1-7D8-K409R
IgG1-2F8-L368VxIgG1-7D8-K409R
IgG1-2F8-L368WxIgG1-7D8-K409R
IgG1-2F8-L368KxIgG1-7D8-K409R
IgG4-2F8xIgG4-7D8
IgG1-2F8-L368FxIgG1-7D8-K409R
IgG1-2F8-L368MxIgG1-7D8-K409R
IgG1-2F8-L368YxIgG1-7D8-K409R
IgG1-2F8xIgG1-7D8-K409R

B

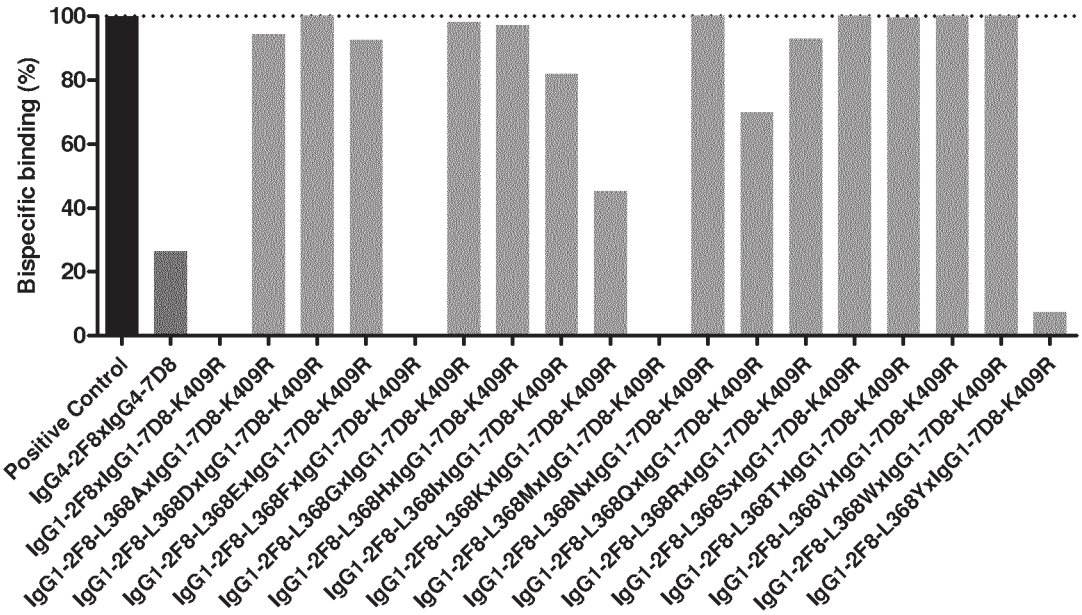
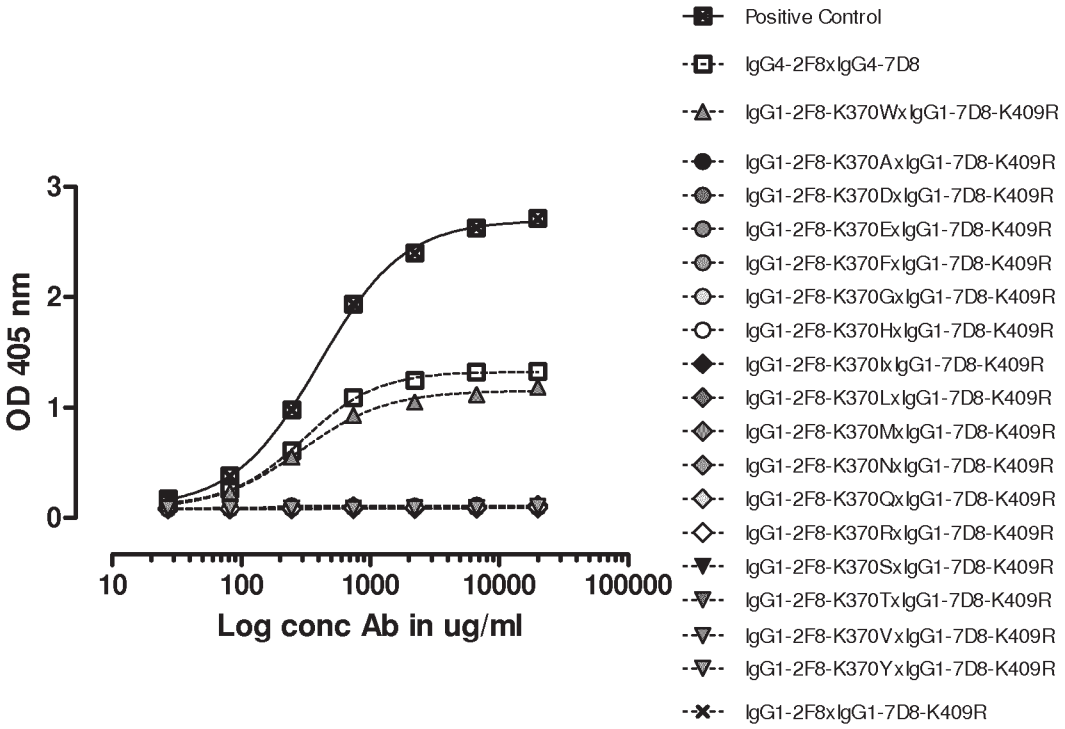

Bispecific binding (%)
100
80
60
40
20
0
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-L368AxIgG1-7D8-K409R
IgG1-2F8-L368DxIgG1-7D8-K409R
IgG1-2F8-L368ExIgG1-7D8-K409R
IgG1-2F8-L368FxIgG1-7D8-K409R
IgG1-2F8-L368GxIgG1-7D8-K409R
IgG1-2F8-L368HxIgG1-7D8-K409R
IgG1-2F8-L368IxIgG1-7D8-K409R
IgG1-2F8-L368KxIgG1-7D8-K409R
IgG1-2F8-L368MxIgG1-7D8-K409R
IgG1-2F8-L368NxIgG1-7D8-K409R
IgG1-2F8-L368QxIgG1-7D8-K409R
IgG1-2F8-L368RxIgG1-7D8-K409R
IgG1-2F8-L368SxIgG1-7D8-K409R
IgG1-2F8-L368TxIgG1-7D8-K409R
IgG1-2F8-L368VxIgG1-7D8-K409R
IgG1-2F8-L368WxIgG1-7D8-K409R
IgG1-2F8-L368YxIgG1-7D8-K409R

Figure 36

A


OD 405 nm
3
2
1
0
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8-K370WxIgG1-7D8-K409R
IgG1-2F8-K370AxIgG1-7D8-K409R
IgG1-2F8-K370DxIgG1-7D8-K409R
IgG1-2F8-K370ExIgG1-7D8-K409R
IgG1-2F8-K370FxIgG1-7D8-K409R
IgG1-2F8-K370GxIgG1-7D8-K409R
IgG1-2F8-K370HxIgG1-7D8-K409R
IgG1-2F8-K370IxIgG1-7D8-K409R
IgG1-2F8-K370LxIgG1-7D8-K409R
IgG1-2F8-K370MxIgG1-7D8-K409R
IgG1-2F8-K370NxIgG1-7D8-K409R
IgG1-2F8-K370QxIgG1-7D8-K409R
IgG1-2F8-K370RxIgG1-7D8-K409R
IgG1-2F8-K370SxIgG1-7D8-K409R
IgG1-2F8-K370TxIgG1-7D8-K409R
IgG1-2F8-K370VxIgG1-7D8-K409R
IgG1-2F8-K370YxIgG1-7D8-K409R
IgG1-2F8xIgG1-7D8-K409R

B

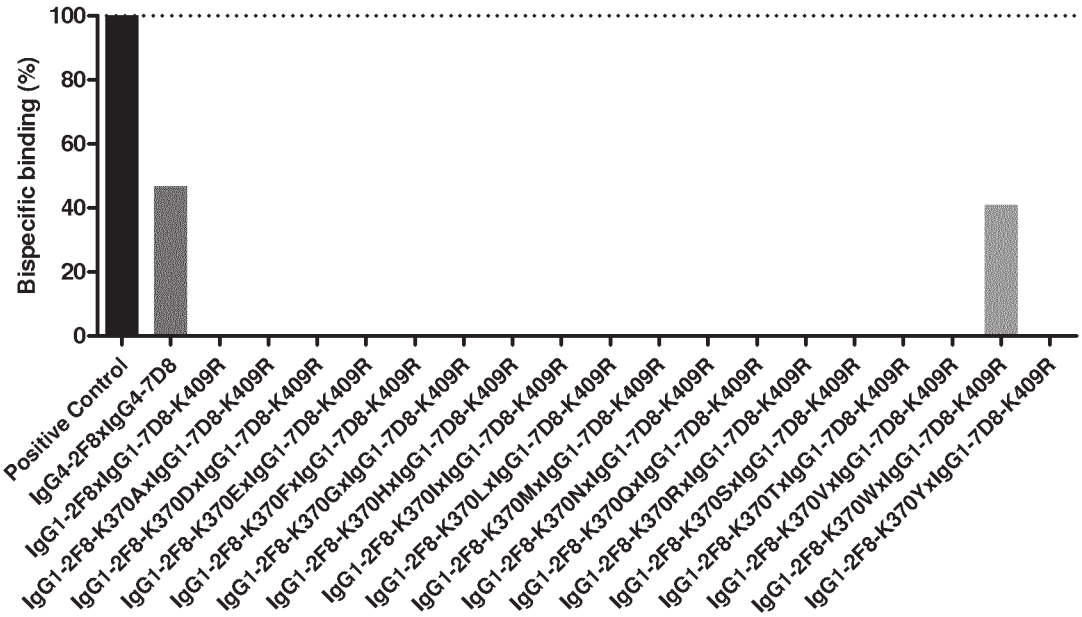
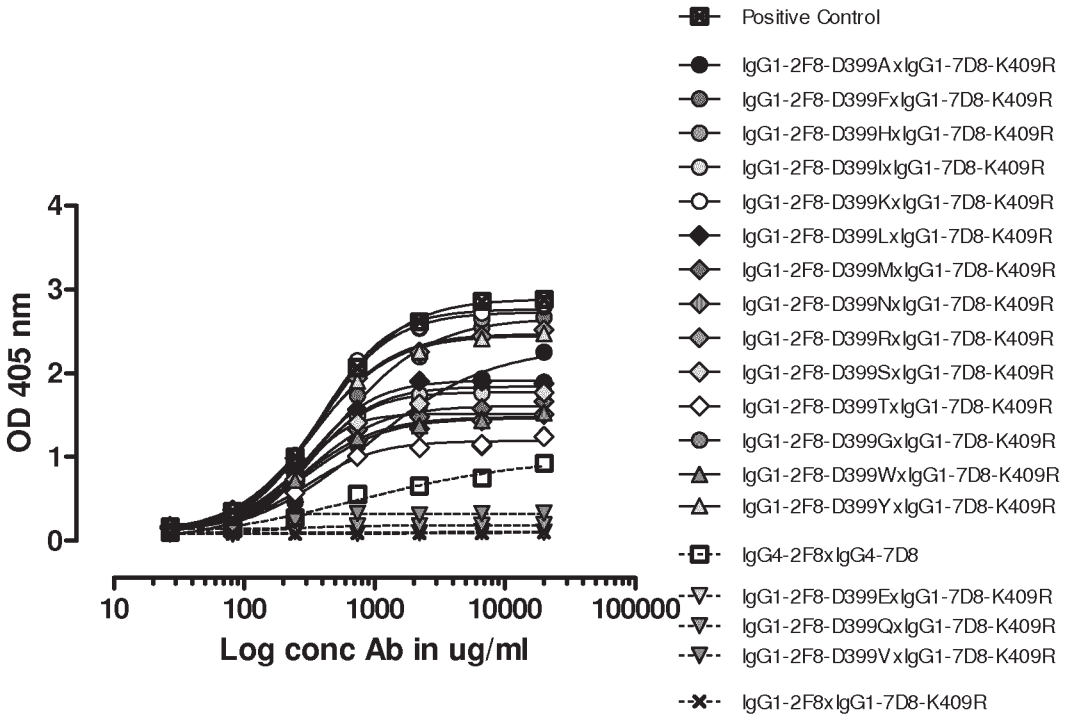

Bispecific binding (%)
100
80
60
40
20
0
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-K370AxIgG1-7D8-K409R
IgG1-2F8-K370DxIgG1-7D8-K409R
IgG1-2F8-K370ExIgG1-7D8-K409R
IgG1-2F8-K370FxIgG1-7D8-K409R
IgG1-2F8-K370GxIgG1-7D8-K409R
IgG1-2F8-K370HxIgG1-7D8-K409R
IgG1-2F8-K370IxIgG1-7D8-K409R
IgG1-2F8-K370LxIgG1-7D8-K409R
IgG1-2F8-K370MxIgG1-7D8-K409R
IgG1-2F8-K370NxIgG1-7D8-K409R
IgG1-2F8-K370QxIgG1-7D8-K409R
IgG1-2F8-K370RxIgG1-7D8-K409R
IgG1-2F8-K370SxIgG1-7D8-K409R
IgG1-2F8-K370TxIgG1-7D8-K409R
IgG1-2F8-K370VxIgG1-7D8-K409R
IgG1-2F8-K370WxIgG1-7D8-K409R
IgG1-2F8-K370YxIgG1-7D8-K409R

Figure 37

A


OD 405 nm
0
1
2
3
4
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG1-2F8-D399AxIgG1-7D8-K409R
IgG1-2F8-D399FxIgG1-7D8-K409R
IgG1-2F8-D399HxIgG1-7D8-K409R
IgG1-2F8-D399IxIgG1-7D8-K409R
IgG1-2F8-D399KxIgG1-7D8-K409R
IgG1-2F8-D399LxIgG1-7D8-K409R
IgG1-2F8-D399MxIgG1-7D8-K409R
IgG1-2F8-D399NxIgG1-7D8-K409R
IgG1-2F8-D399RxIgG1-7D8-K409R
IgG1-2F8-D399SxIgG1-7D8-K409R
IgG1-2F8-D399TxIgG1-7D8-K409R
IgG1-2F8-D399GxIgG1-7D8-K409R
IgG1-2F8-D399WxIgG1-7D8-K409R
IgG1-2F8-D399YxIgG1-7D8-K409R
IgG4-2F8xIgG4-7D8
IgG1-2F8-D399ExIgG1-7D8-K409R
IgG1-2F8-D399QxIgG1-7D8-K409R
IgG1-2F8-D399VxIgG1-7D8-K409R
IgG1-2F8xIgG1-7D8-K409R

B

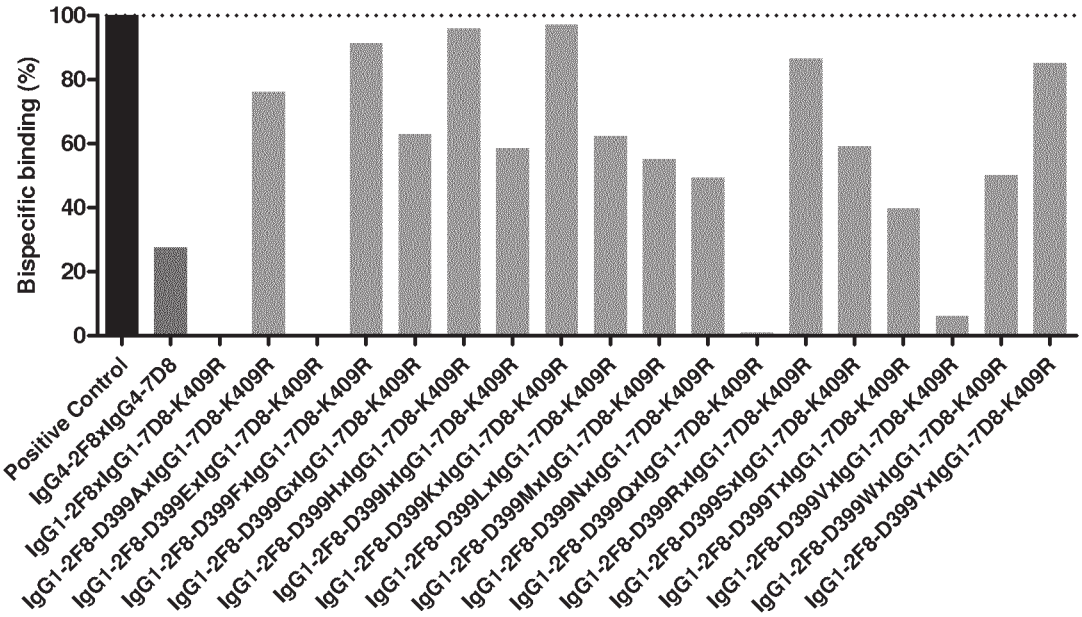
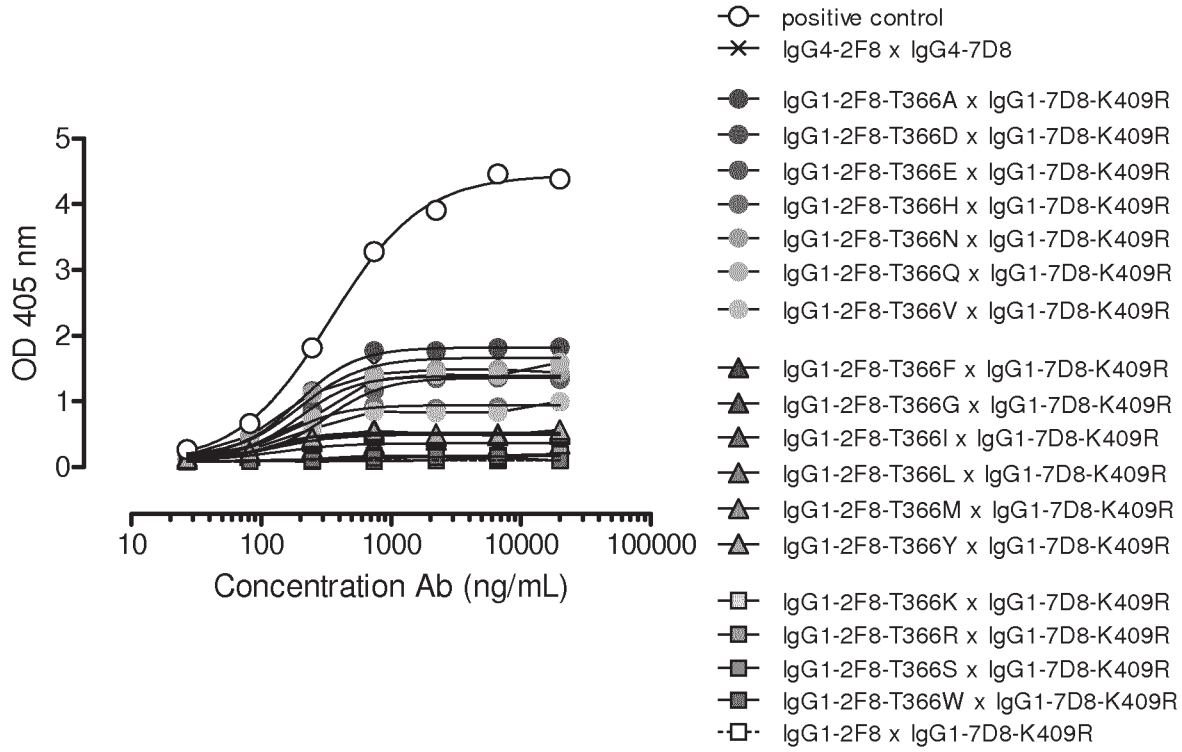

Bispecific binding (%)
0
20
40
60
80
100
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-D399AxIgG1-7D8-K409R
IgG1-2F8-D399ExIgG1-7D8-K409R
IgG1-2F8-D399FxIgG1-7D8-K409R
IgG1-2F8-D399GxIgG1-7D8-K409R
IgG1-2F8-D399HxIgG1-7D8-K409R
IgG1-2F8-D399IxIgG1-7D8-K409R
IgG1-2F8-D399KxIgG1-7D8-K409R
IgG1-2F8-D399LxIgG1-7D8-K409R
IgG1-2F8-D399MxIgG1-7D8-K409R
IgG1-2F8-D399NxIgG1-7D8-K409R
IgG1-2F8-D399QxIgG1-7D8-K409R
IgG1-2F8-D399RxIgG1-7D8-K409R
IgG1-2F8-D399SxIgG1-7D8-K409R
IgG1-2F8-D399TxIgG1-7D8-K409R
IgG1-2F8-D399VxIgG1-7D8-K409R
IgG1-2F8-D399WxIgG1-7D8-K409R
IgG1-2F8-D399YxIgG1-7D8-K409R

Figure 38

A


OD 405 nm
0
1
2
3
4
5
10
100
1000
10000
100000
Concentration Ab (ng/mL)
positive control
IgG4-2F8 x IgG4-7D8
IgG1-2F8-T366A x IgG1-7D8-K409R
IgG1-2F8-T366D x IgG1-7D8-K409R
IgG1-2F8-T366E x IgG1-7D8-K409R
IgG1-2F8-T366H x IgG1-7D8-K409R
IgG1-2F8-T366N x IgG1-7D8-K409R
IgG1-2F8-T366Q x IgG1-7D8-K409R
IgG1-2F8-T366V x IgG1-7D8-K409R
IgG1-2F8-T366F x IgG1-7D8-K409R
IgG1-2F8-T366G x IgG1-7D8-K409R
IgG1-2F8-T366I x IgG1-7D8-K409R
IgG1-2F8-T366L x IgG1-7D8-K409R
IgG1-2F8-T366M x IgG1-7D8-K409R
IgG1-2F8-T366Y x IgG1-7D8-K409R
IgG1-2F8-T366K x IgG1-7D8-K409R
IgG1-2F8-T366R x IgG1-7D8-K409R
IgG1-2F8-T366S x IgG1-7D8-K409R
IgG1-2F8-T366W x IgG1-7D8-K409R
IgG1-2F8 x IgG1-7D8-K409R

B

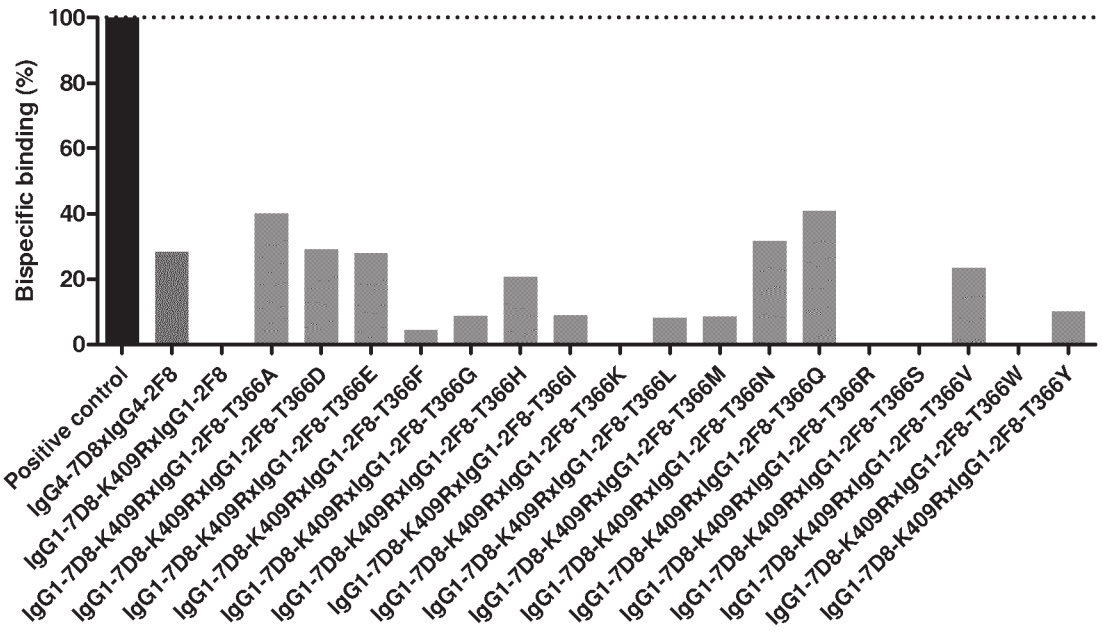

Bispecific binding (%)
0
20
40
60
80
100
Positive control
IgG4-7D8xIgG4-2F8
IgG1-7D8-K409RxIgG1-2F8
IgG1-7D8-K409RxIgG1-2F8-T366A
IgG1-7D8-K409RxIgG1-2F8-T366D
IgG1-7D8-K409RxIgG1-2F8-T366E
IgG1-7D8-K409RxIgG1-2F8-T366F
IgG1-7D8-K409RxIgG1-2F8-T366G
IgG1-7D8-K409RxIgG1-2F8-T366H
IgG1-7D8-K409RxIgG1-2F8-T366I
IgG1-7D8-K409RxIgG1-2F8-T366K
IgG1-7D8-K409RxIgG1-2F8-T366L
IgG1-7D8-K409RxIgG1-2F8-T366M
IgG1-7D8-K409RxIgG1-2F8-T366N
IgG1-7D8-K409RxIgG1-2F8-T366Q
IgG1-7D8-K409RxIgG1-2F8-T366R
IgG1-7D8-K409RxIgG1-2F8-T366S
IgG1-7D8-K409RxIgG1-2F8-T366V
IgG1-7D8-K409RxIgG1-2F8-T366W
IgG1-7D8-K409RxIgG1-2F8-T366Y

Figure 39

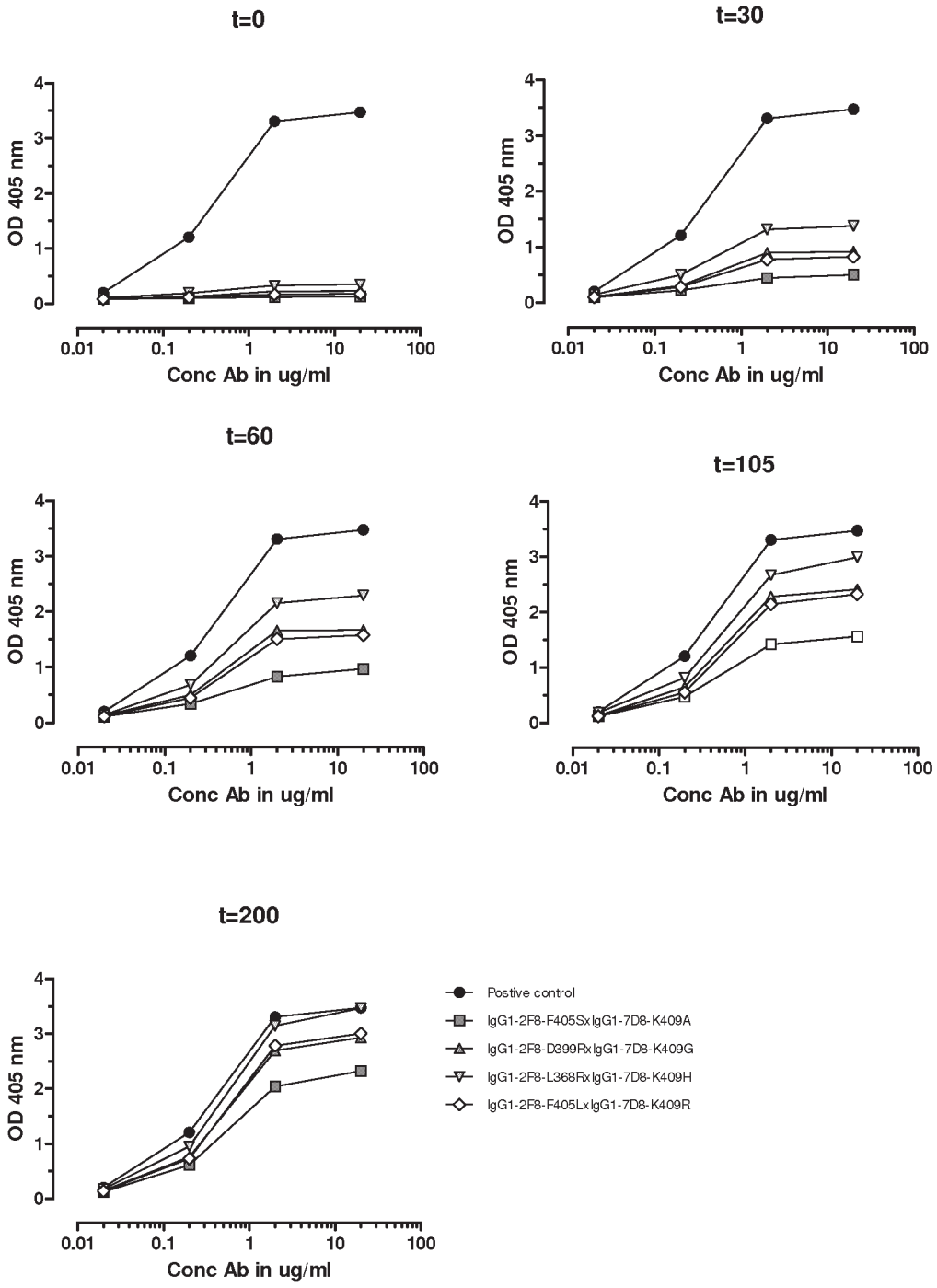

t=0
t=30
t=60
t=105
t=200
OD 405 nm
Conc Ab in ug/ml
0.01
0.1
1
10
100
0
1
2
3
4
Postive control
IgG1-2F8-F405SxIgG1-7D8-K409A
IgG1-2F8-D399RxIgG1-7D8-K409G
IgG1-2F8-L368RxIgG1-7D8-K409H
IgG1-2F8-F405LxIgG1-7D8-K409R

Figure 40

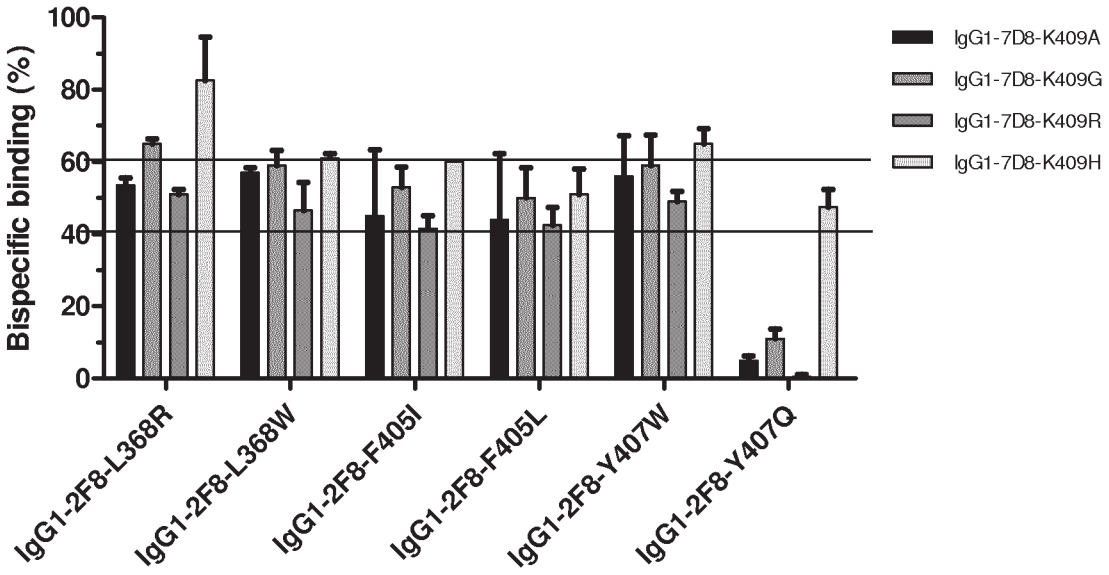

Bispecific binding (%)
100
80
60
40
20
0
IgG1-7D8-K409A
IgG1-7D8-K409G
IgG1-7D8-K409R
IgG1-7D8-K409H
IgG1-2F8-L368R
IgG1-2F8-L368W
IgG1-2F8-F405I
IgG1-2F8-F405L
IgG1-2F8-Y407W
IgG1-2F8-Y407Q

Figure 41

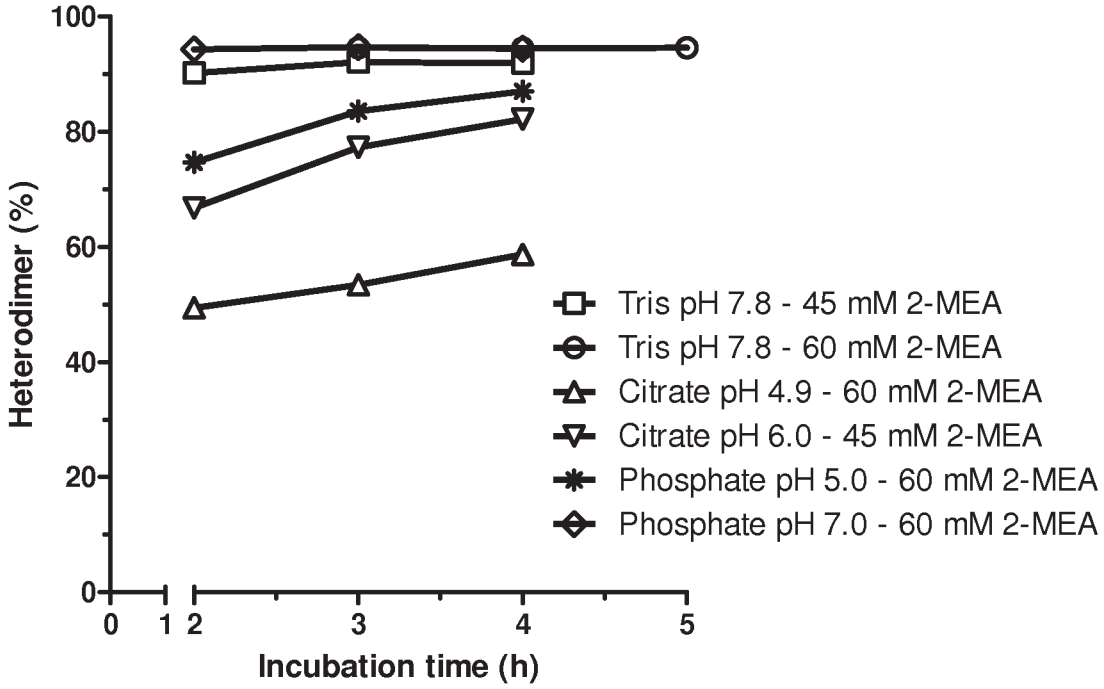

100
80
60
40
20
0
Heterodimer (%)
0
1
2
3
4
5
Incubation time (h)
Tris pH 7.8 - 45 mM 2-MEA
Tris pH 7.8 - 60 mM 2-MEA
Citrate pH 4.9 - 60 mM 2-MEA
Citrate pH 6.0 - 45 mM 2-MEA
Phosphate pH 5.0 - 60 mM 2-MEA
Phosphate pH 7.0 - 60 mM 2-MEA

Figure 42

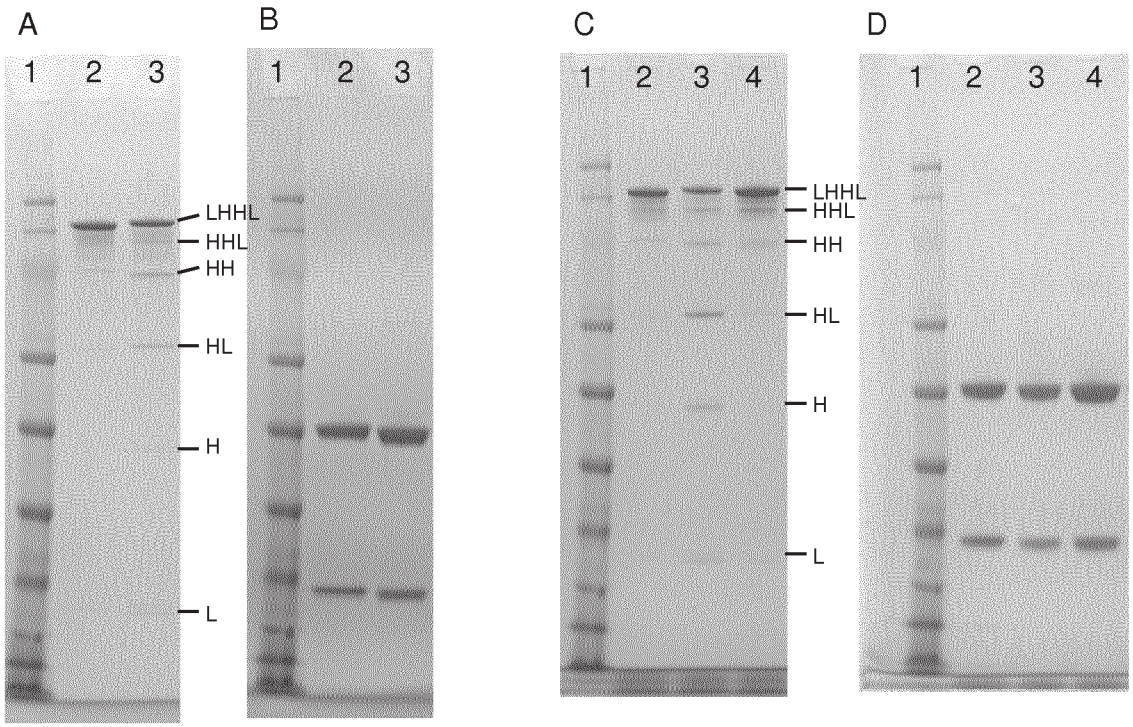


Figure 43

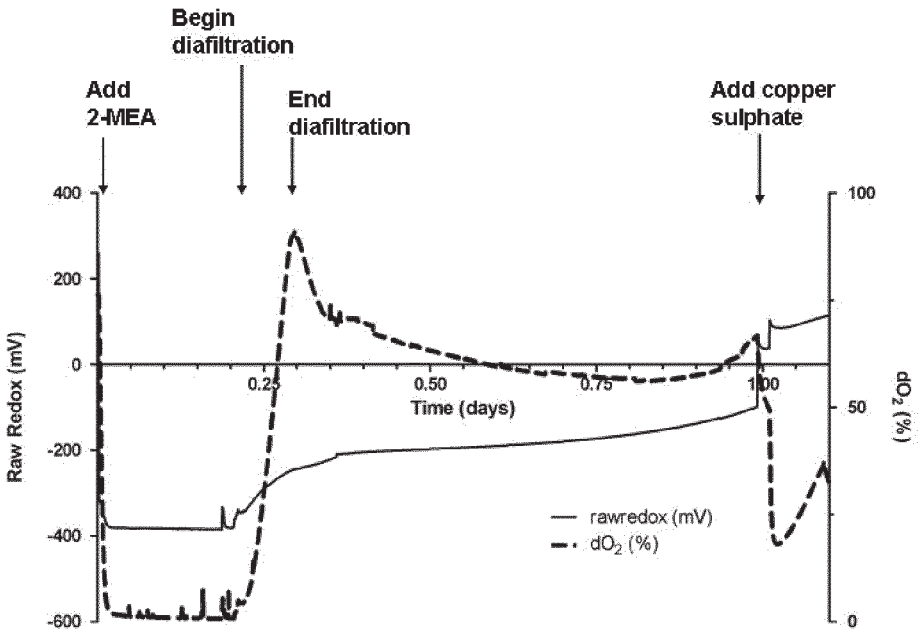

Begin diafiltration
Add 2-MEA
End diafiltration
Add copper sulphate
400
200
0
-200
-400
-600
Raw Redox (mV)
0.25
0.50
0.75
1.00
Time (days)
100
50
0
dO2 (%)
rawredox (mV)
dO2 (%)

Figure 44

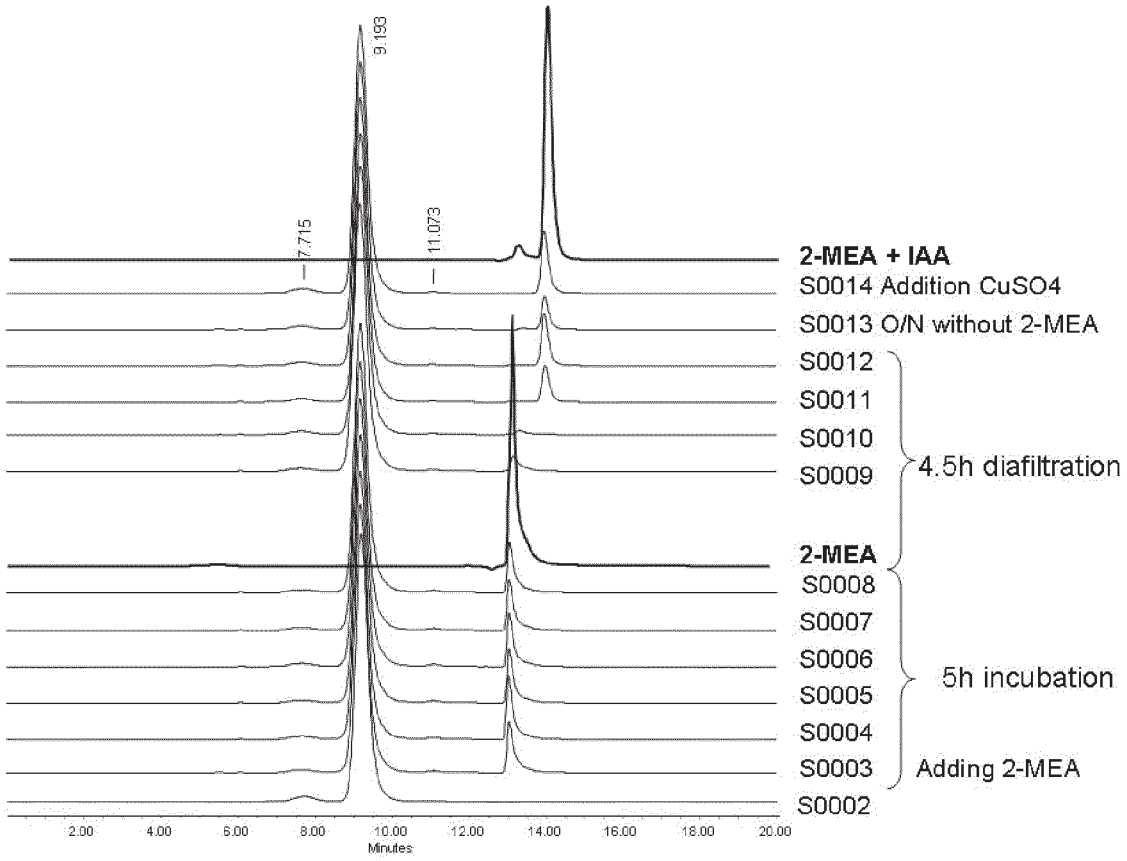

9.193
7.715
11.073
2-MEA + IAA
S0014 Addition CuSO4
S0013 O/N without 2-MEA
S0012
S0011
S0010
S0009
4.5h diafiltration
2-MEA
S0008
S0007
S0006
S0005
5h incubation
S0004
S0003
Adding 2-MEA
S0002
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

Figure 45

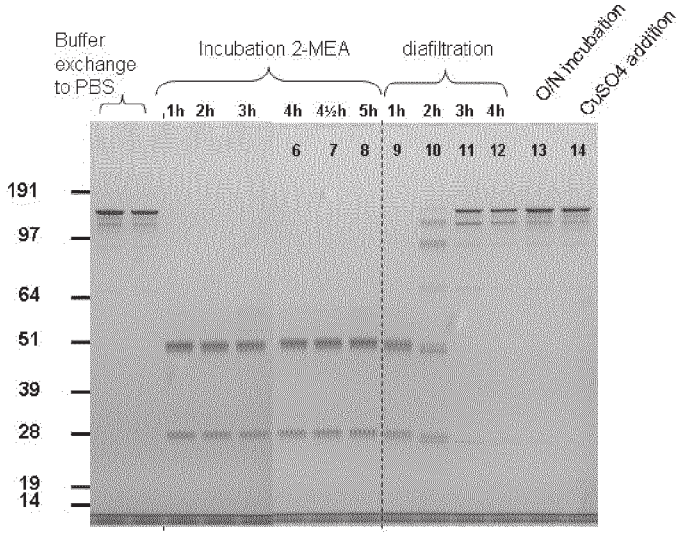

Buffer exchange to PBS
Incubation 2-MEA
diafiltration
O/N incubation
CuSO4 addition
1h 2h 3h 4h 4½h 5h 1h 2h 3h 4h
6 7 8 9 10 11 12 13 14
191
97
64
51
39
28
19
14

Figure 46

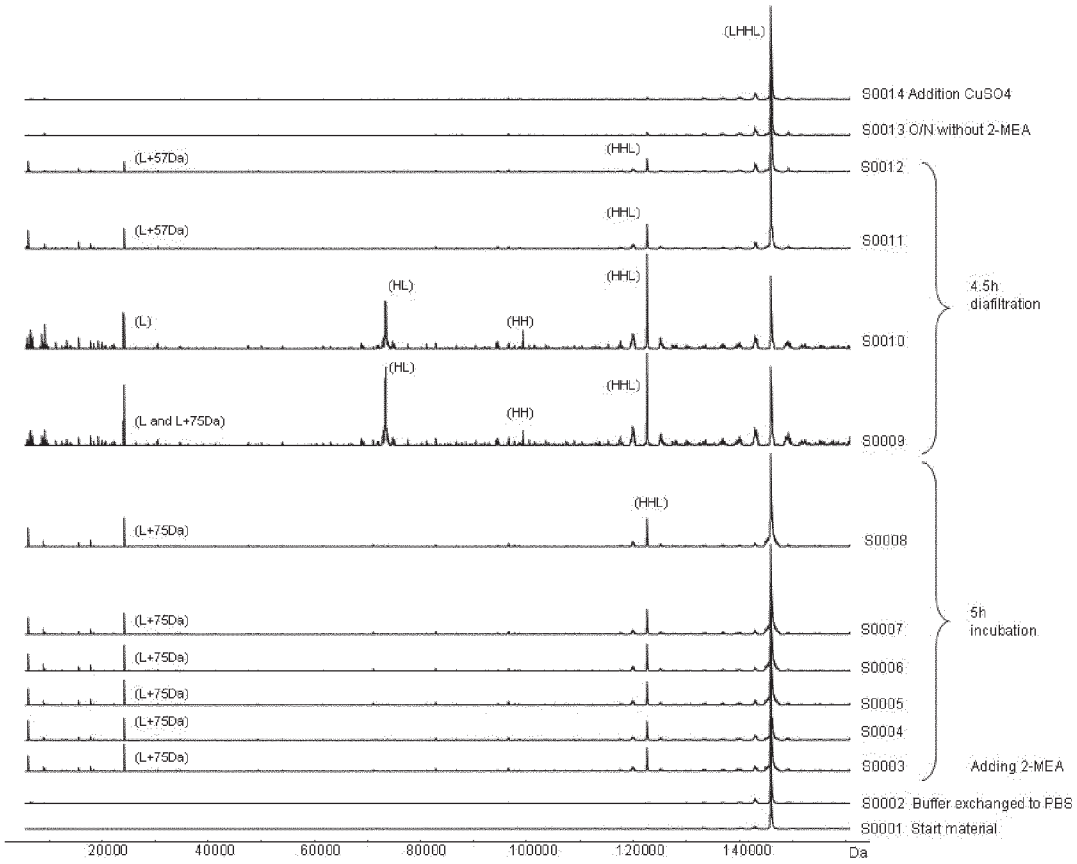

(LHHL)
S0014 Addition CuSO4
S0013 O/N without 2-MEA
(L+57Da)
(HHL)
S0012
(L+57Da)
(HHL)
S0011
(L)
(HL)
(HH)
(HHL)
S0010
4.5h diafiltration
(L and L+75Da)
(HL)
(HH)
(HHL)
S0009
(L+75Da)
(HHL)
S0008
(L+75Da)
S0007
5h incubation
(L+75Da)
S0006
(L+75Da)
S0005
(L+75Da)
S0004
(L+75Da)
S0003
Adding 2-MEA
S0002 Buffer exchanged to PBS
S0001 Start material
20000
40000
60000
80000
100000
120000
140000
Da

Figure 47

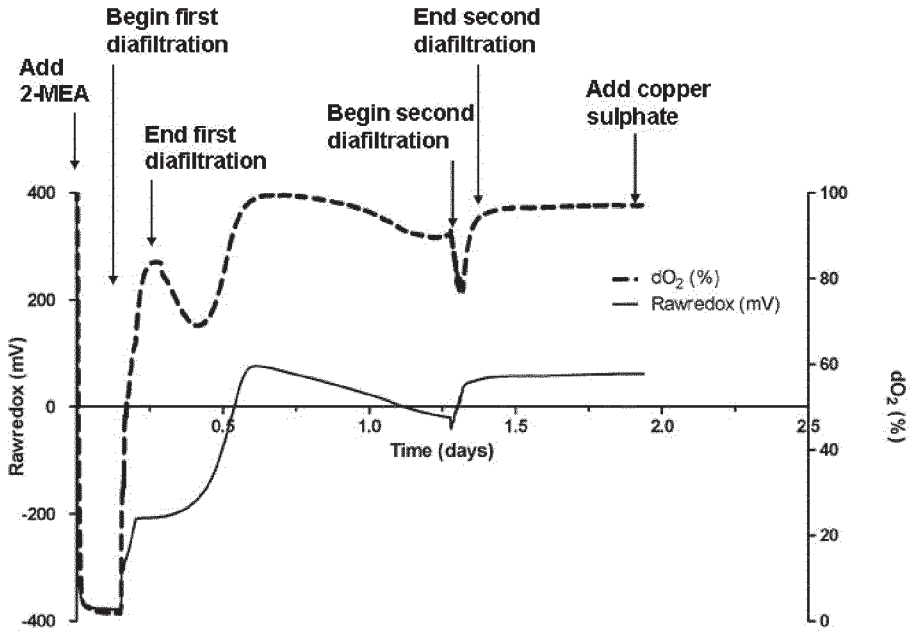

Add 2-MEA
Begin first diafiltration
End first diafiltration
Begin second diafiltration
End second diafiltration
Add copper sulphate
400
200
0
-200
-400
Rawredox (mV)
0.5
1.0
1.5
2.0
2.5
Time (days)
100
80
60
40
20
0
dO2 (%)
dO2 (%)
Rawredox (mV)

Figure 48

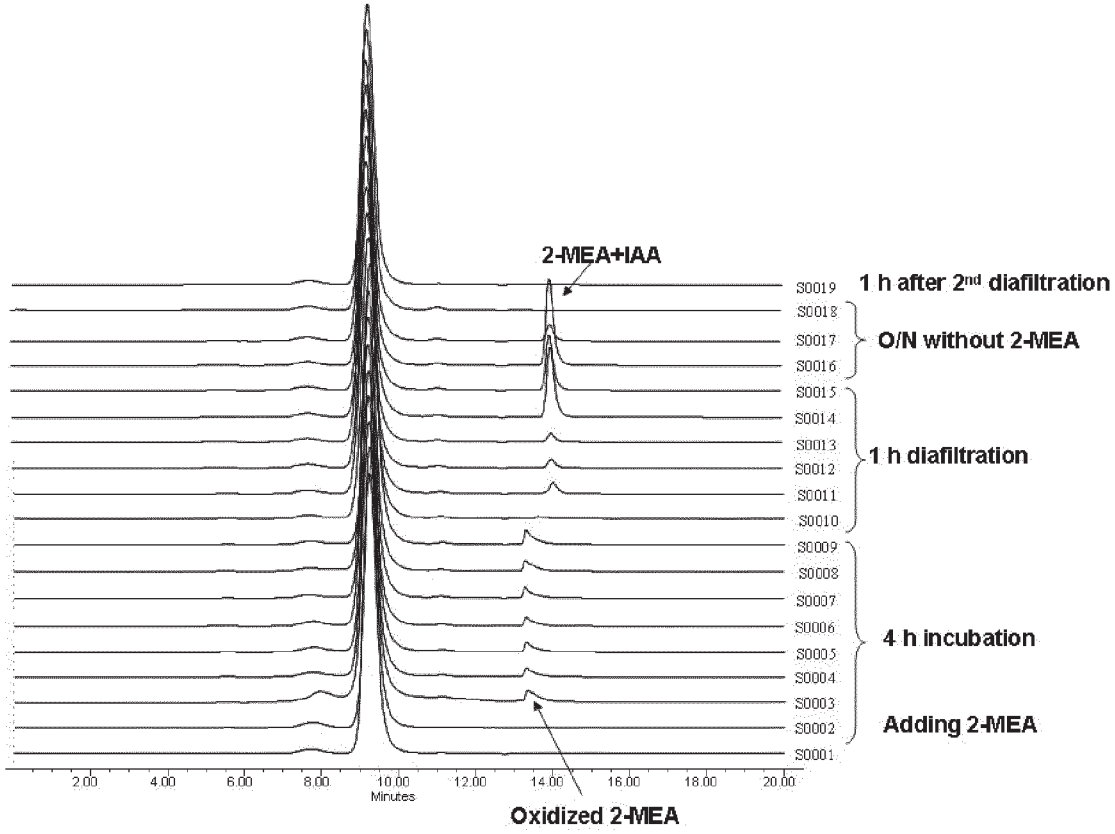

2-MEA+IAA
S0019
S0018
S0017
S0016
S0015
S0014
S0013
S0012
S0011
S0010
S0009
S0008
S0007
S0006
S0005
S0004
S0003
S0002
S0001
1 h after 2nd diafiltration
O/N without 2-MEA
1 h diafiltration
4 h incubation
Adding 2-MEA
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes
Oxidized 2-MEA

Figure 49

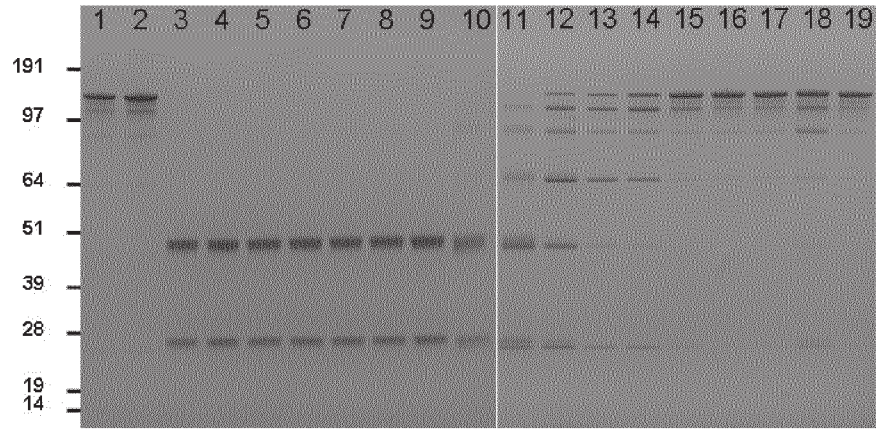


Figure 50

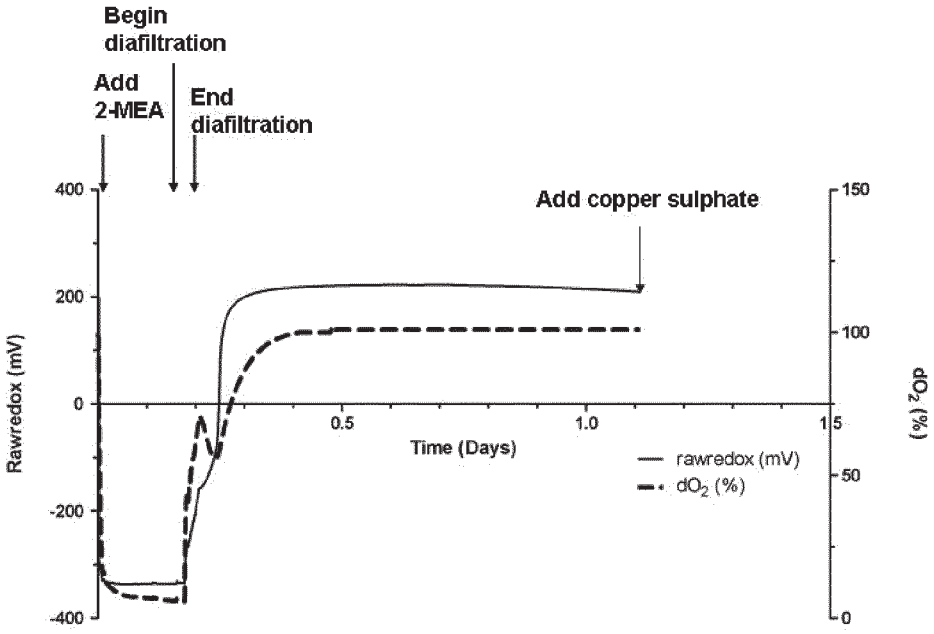

Begin diafiltration
Add 2-MEA
End diafiltration
Add copper sulphate
Rawredox (mV)
400
200
0
-200
-400
0.5
1.0
1.5
Time (Days)
150
100
50
0
(%) ^{2}Op
rawredox (mV)
dO_2 (%)

Figure 51

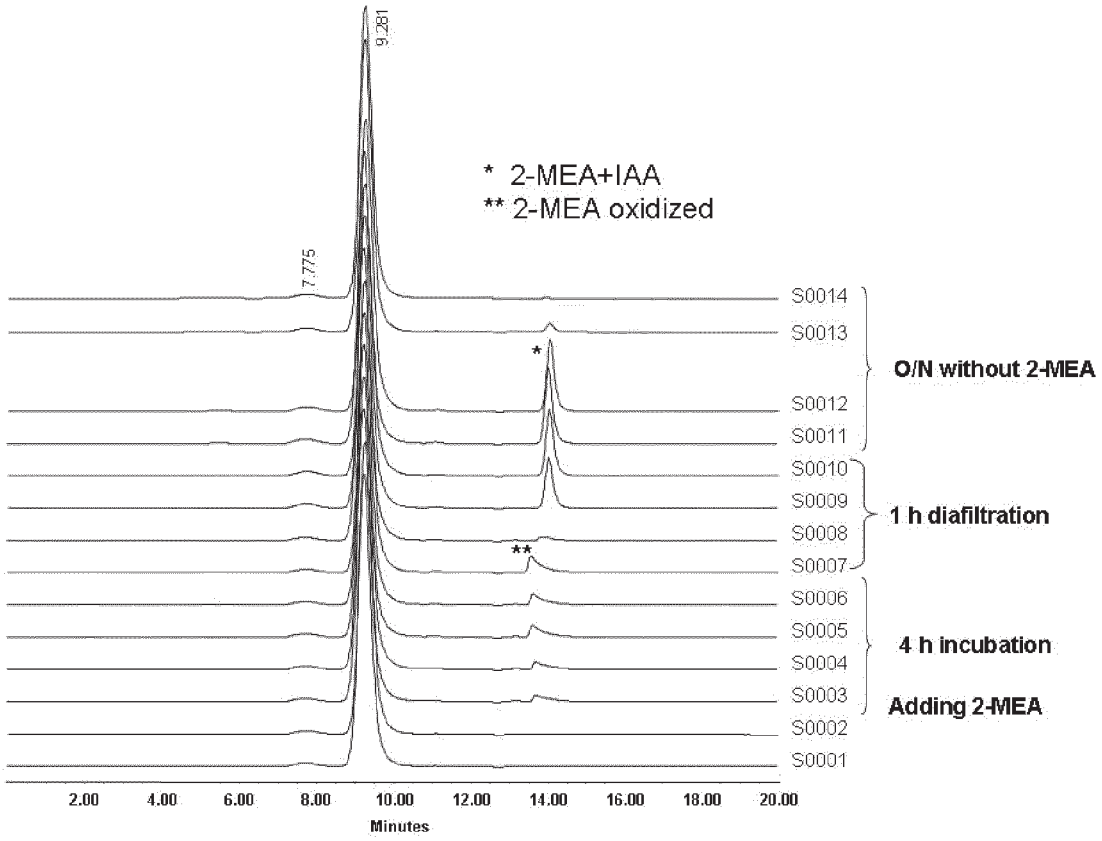

9.281
7.775
* 2-MEA+IAA
** 2-MEA oxidized
S0014
S0013
S0012
S0011
S0010
S0009
S0008
S0007
S0006
S0005
S0004
S0003
S0002
S0001
O/N without 2-MEA
1 h diafiltration
4 h incubation
Adding 2-MEA
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

Figure 52

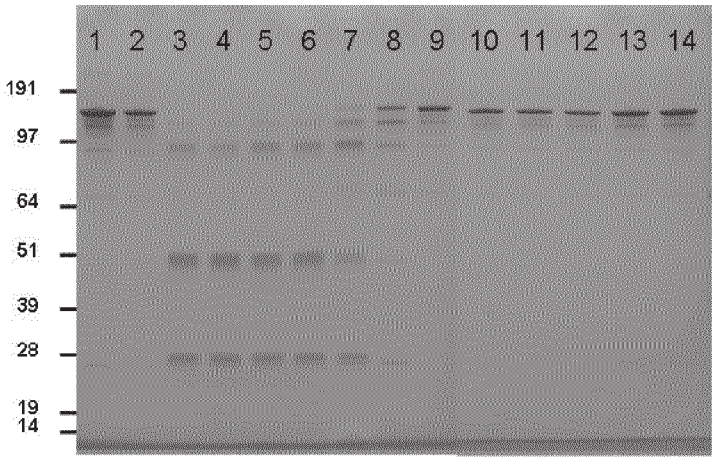
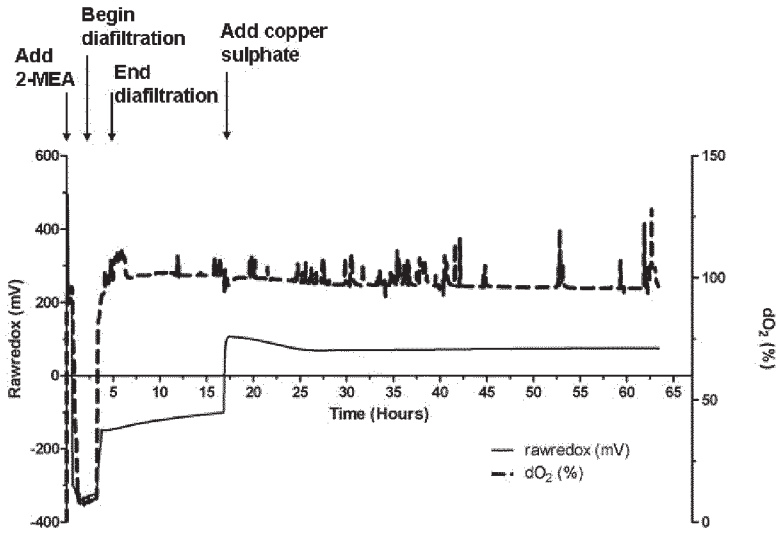


Figure 53

A)


Add 2-MEA
Begin diafiltration
End diafiltration
Add copper sulphate
Rawredox (mV)
dO2 (%)
Time (Hours)
rawredox (mV)
dO2 (%)

B)

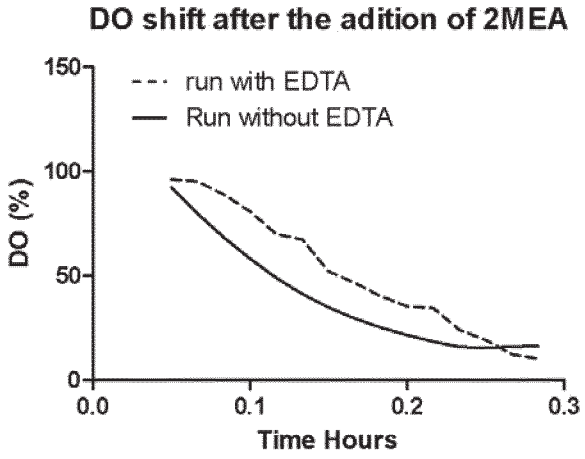

DO shift after the adition of 2MEA
run with EDTA
Run without EDTA
DO (%)
Time Hours

Figure 54

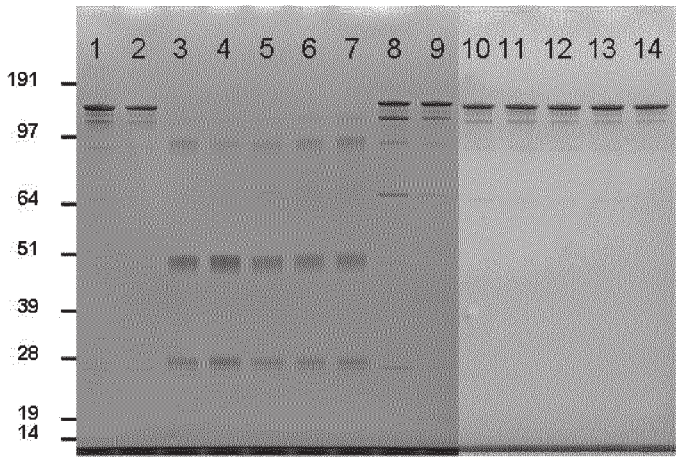


Figure 55

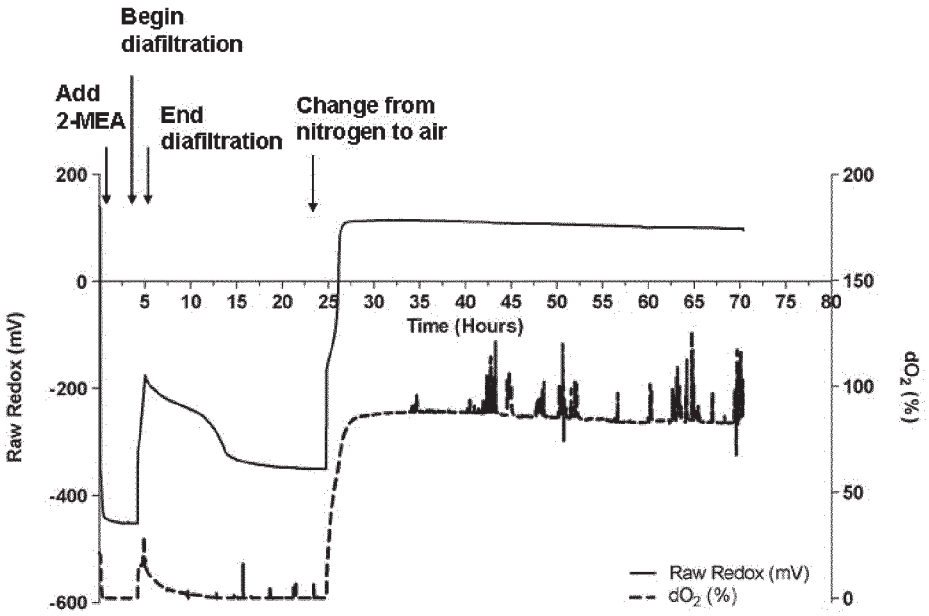

Begin diafiltration
Add 2-MEA
End diafiltration
Change from nitrogen to air
Raw Redox (mV)
Time (Hours)
dO2 (%)
Raw Redox (mV)
dO2 (%)

Figure 56

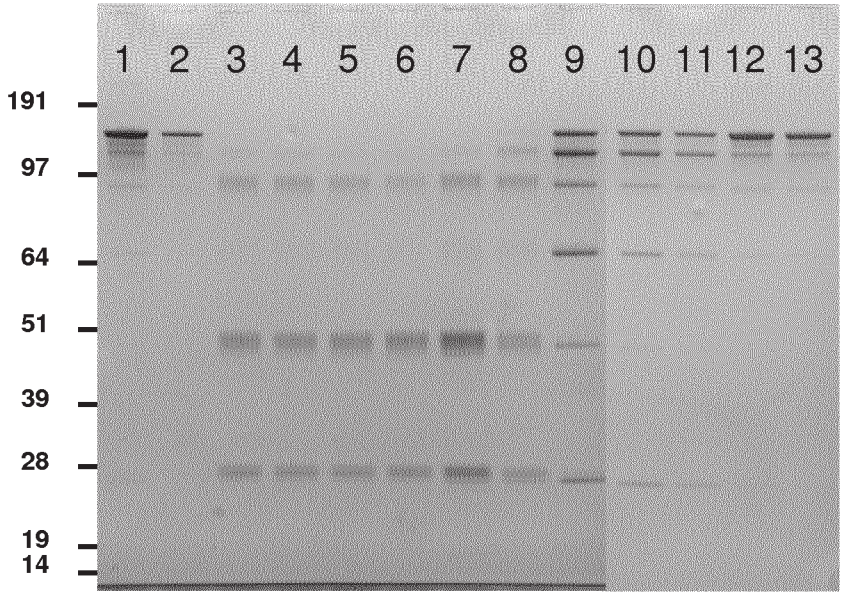


Figure 57

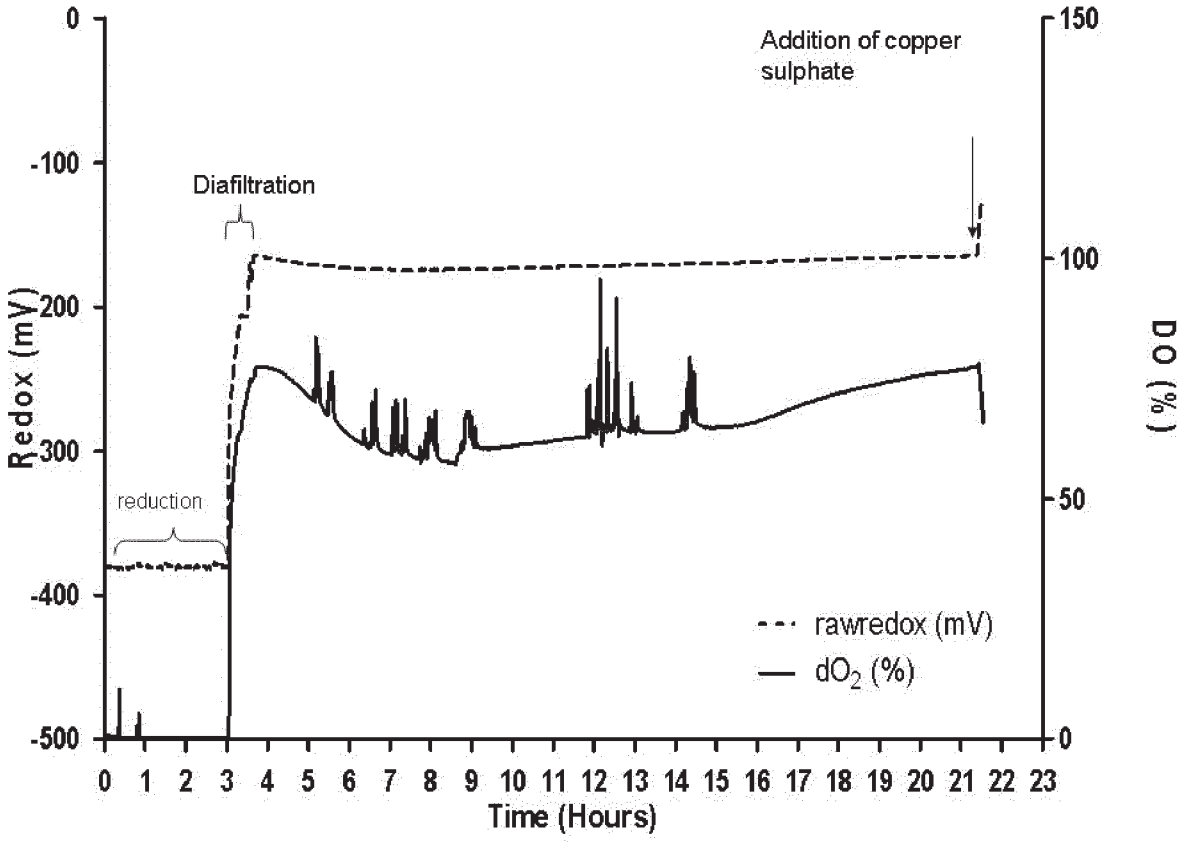

Addition of copper sulphate
Diafiltration
reduction
Redox (mV)
DO (%)
rawredox (mV)
dO2 (%)
Time (Hours)
0
-100
-200
-300
-400
-500
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Figure 58

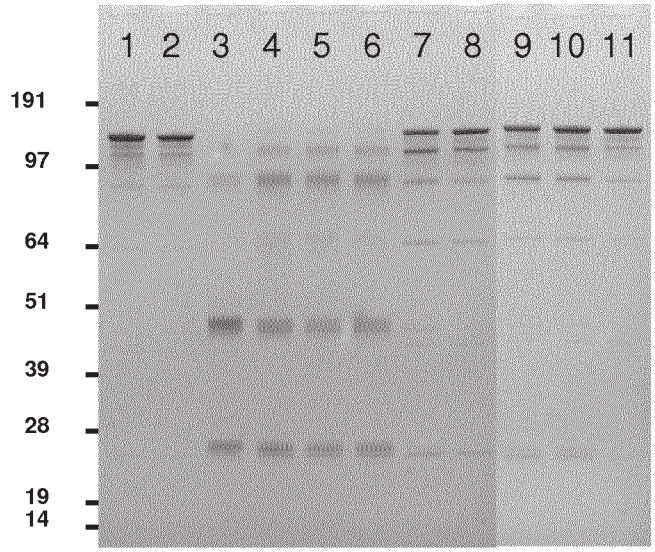


Figure 59

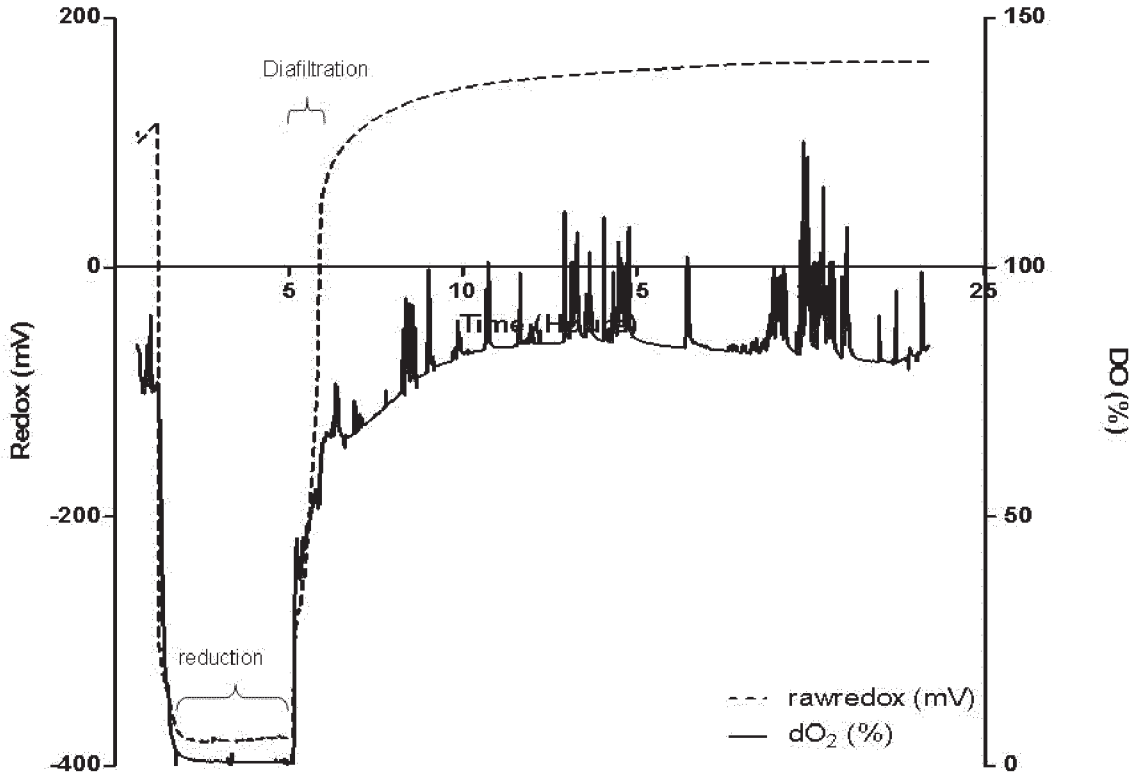

200
0
-200
-400
150
100
50
0
Diafiltration
reduction
5
10
5
25
Redox (mV)
DO (%)
rawredox (mV)
dO2 (%)

Figure 60

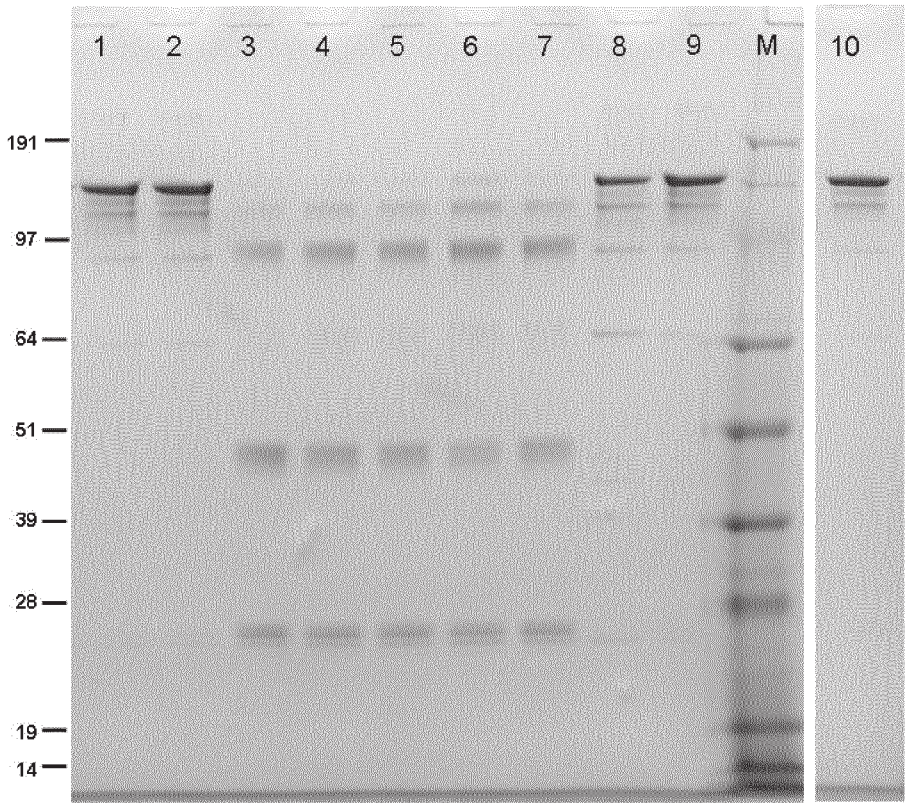


Figure 61

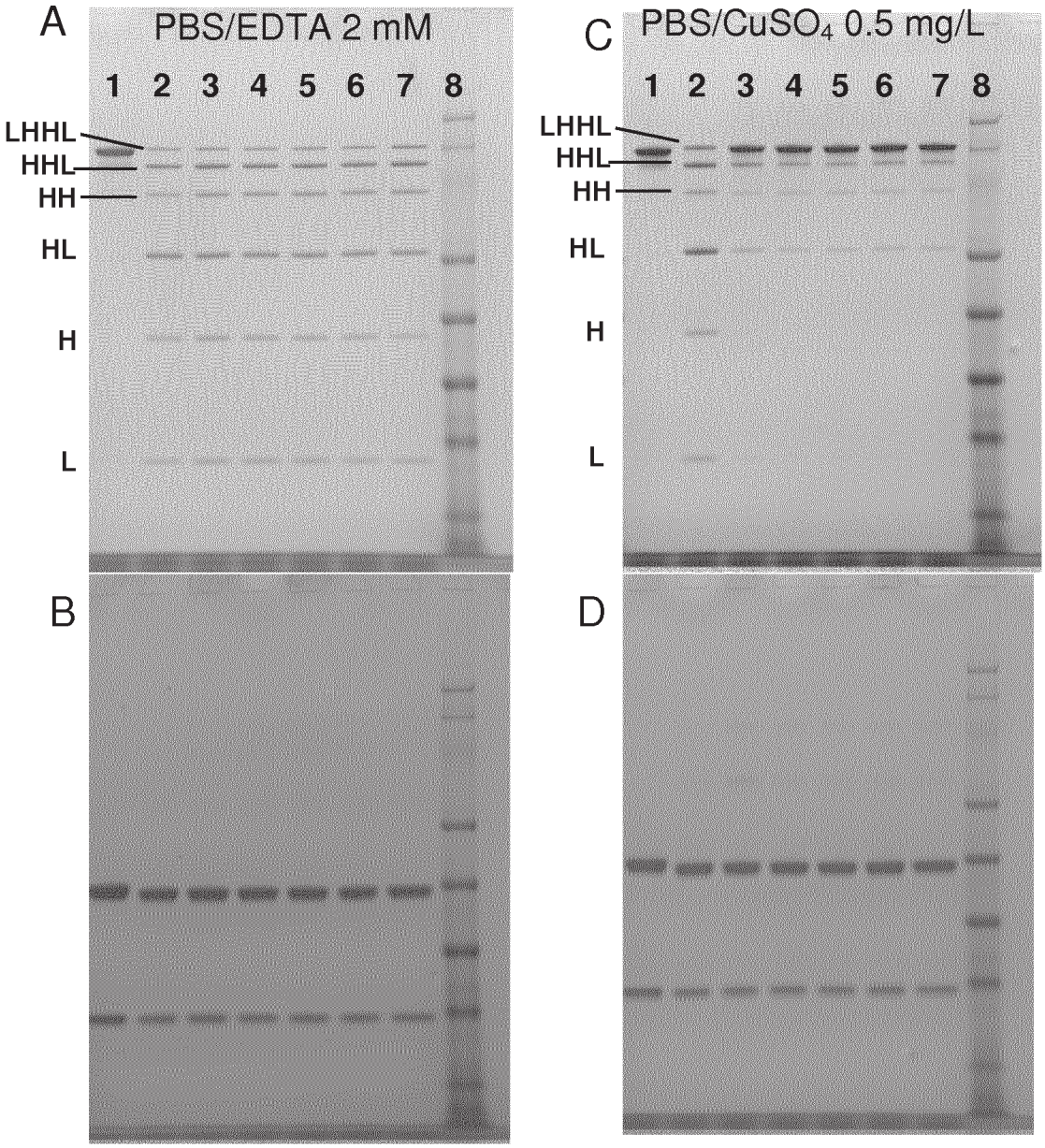
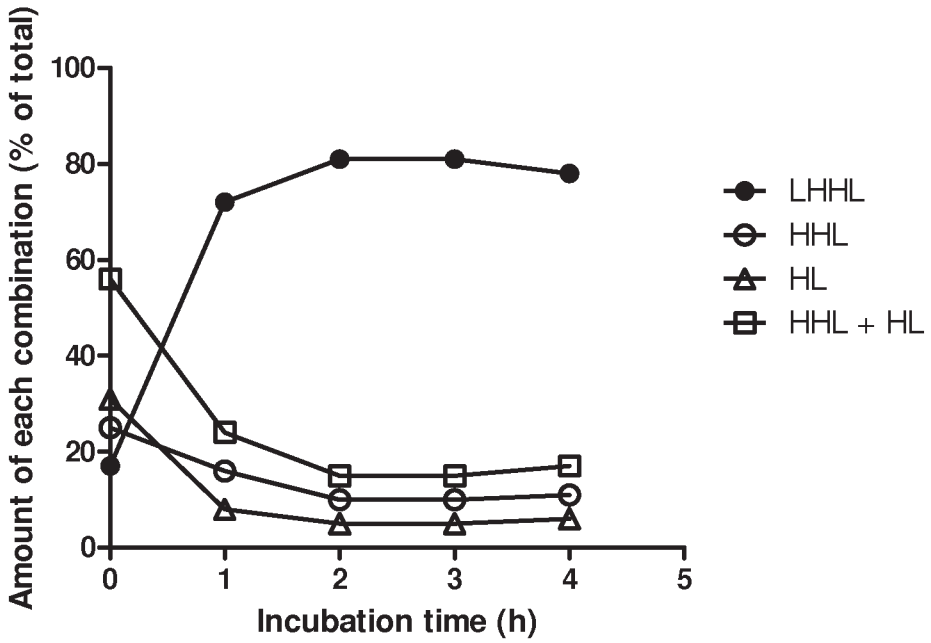

A
PBS/EDTA 2 mM
1 2 3 4 5 6 7 8
LHHL
HHL
HH
HL
H
L
B
C
PBS/CuSO4 0.5 mg/L
1 2 3 4 5 6 7 8
LHHL
HHL
HH
HL
H
L
D

Figure 62


Amount of each combination (% of total)
100
80
60
40
20
0
0
1
2
3
4
5
Incubation time (h)
LHHL
HHL
HL
HHL + HL

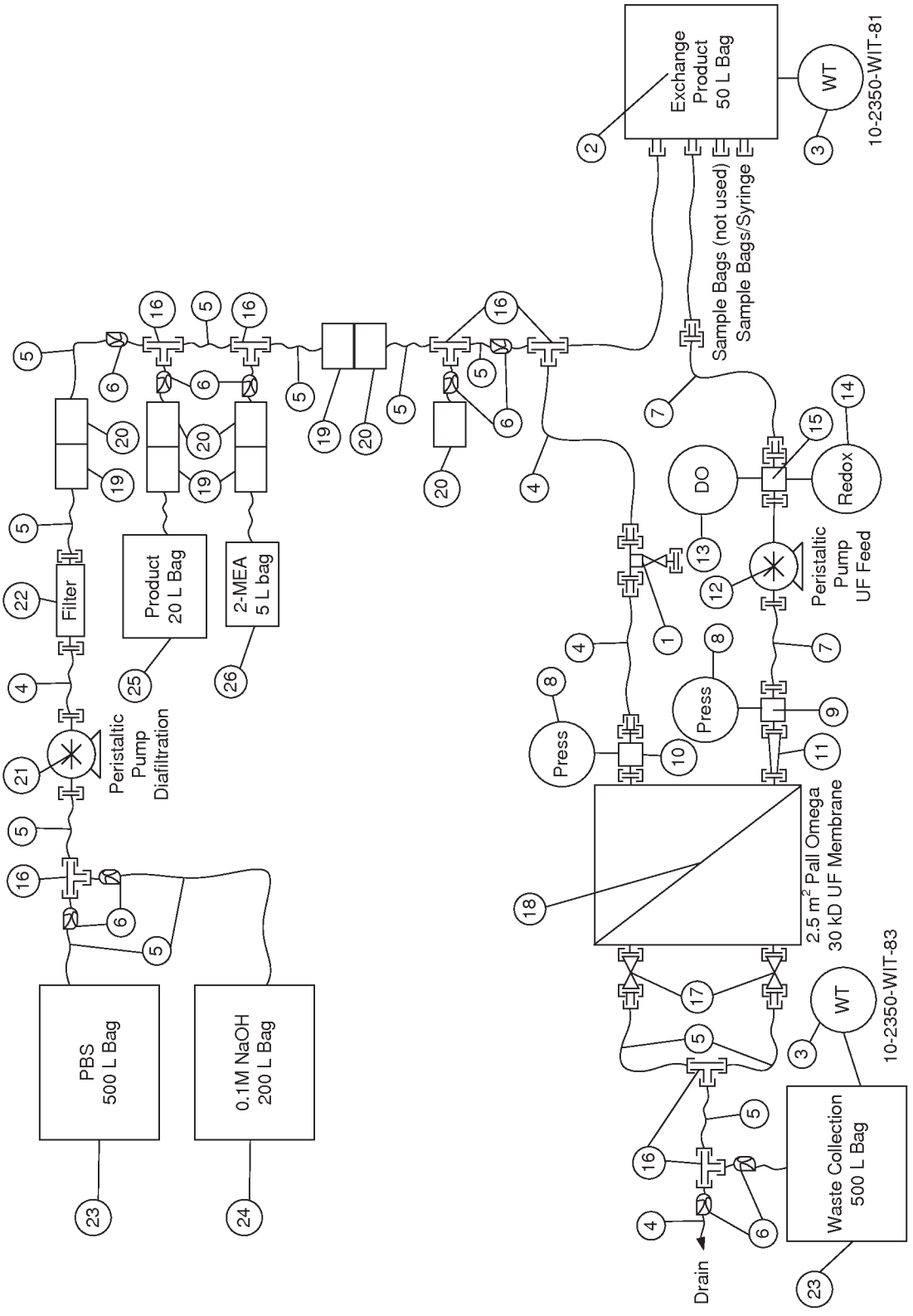

PBS
500 L Bag
0.1M NaOH
200 L Bag
Peristaltic
Pump
Diafiltration
Filter
Product
20 L Bag
2-MEA
5 L bag
Exchange
Product
50 L Bag
WT
10-2350-WIT-81
Sample Bags (not used)
Sample Bags/Syringe
DO
Redox
Peristaltic
Pump
UF Feed
Press
Press
2.5 m2 Pall Omega
30 kD UF Membrane
WT
10-2350-WIT-83
Waste Collection
500 L Bag
Drain

Figure 63

Figure 64

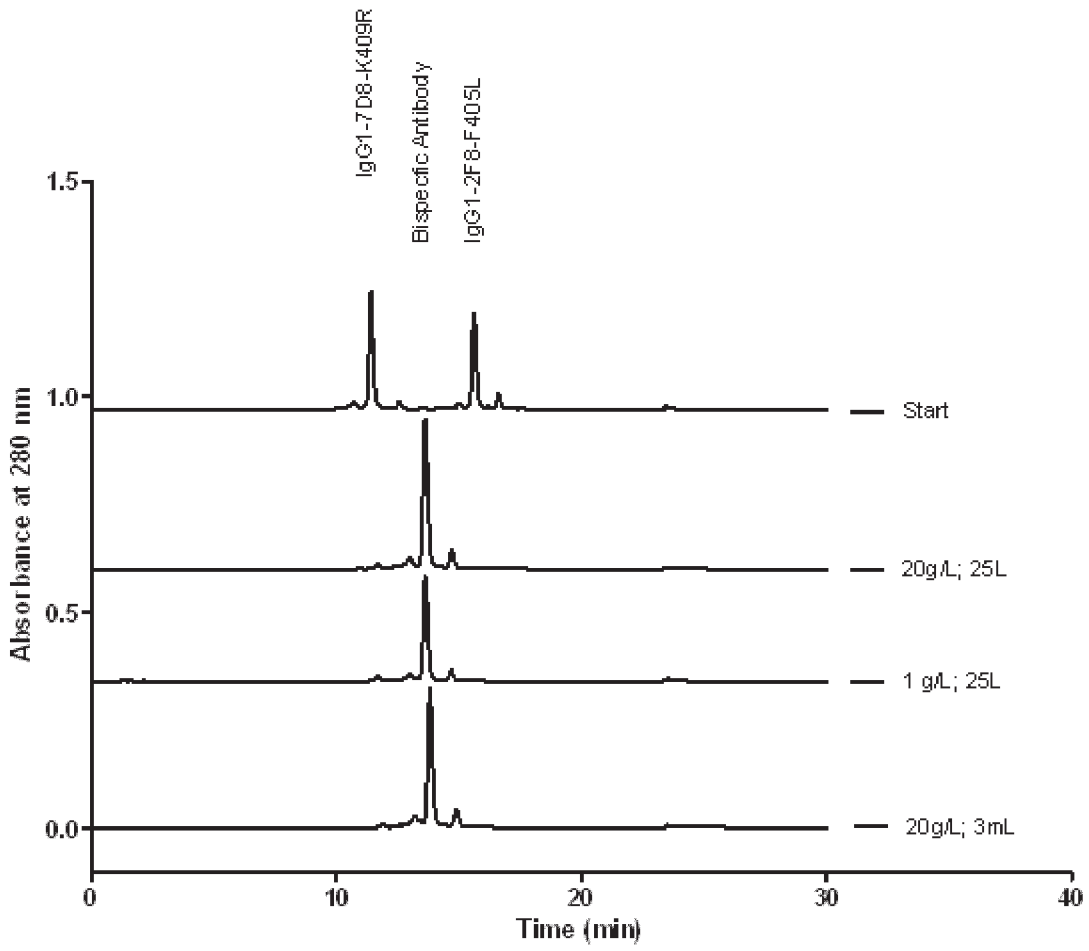

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
1.5
1.0
0.5
0.0
Absorbance at 280 nm
Start
20g/L; 25L
1 g/L; 25L
20g/L; 3mL
0
10
20
30
40
Time (min)

Figure 65

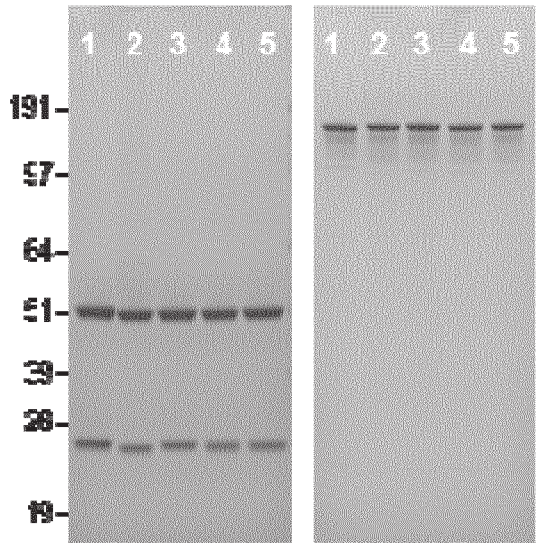


Figure 66

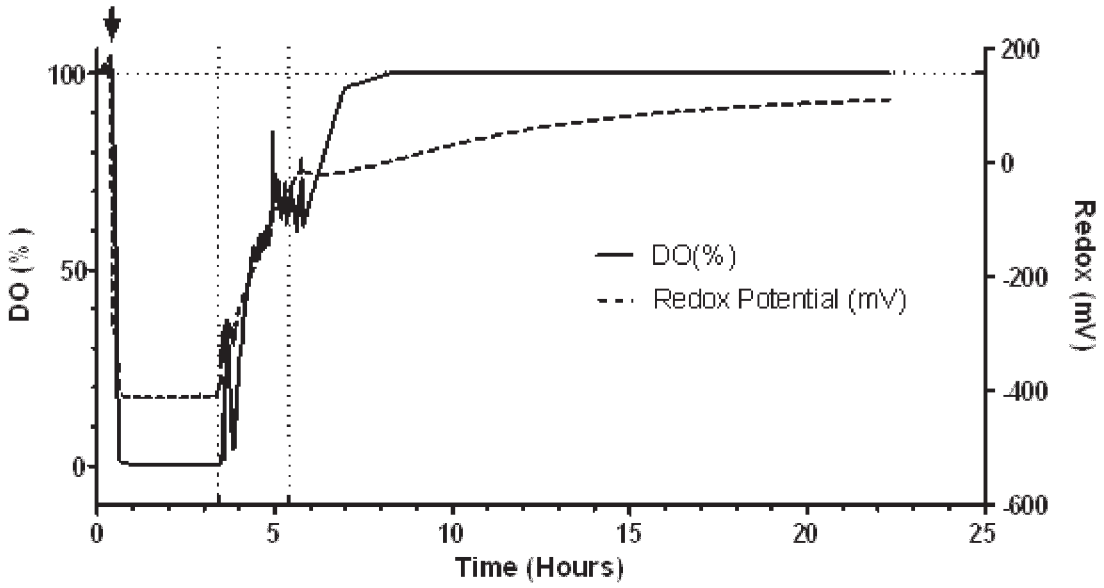


Figure 67

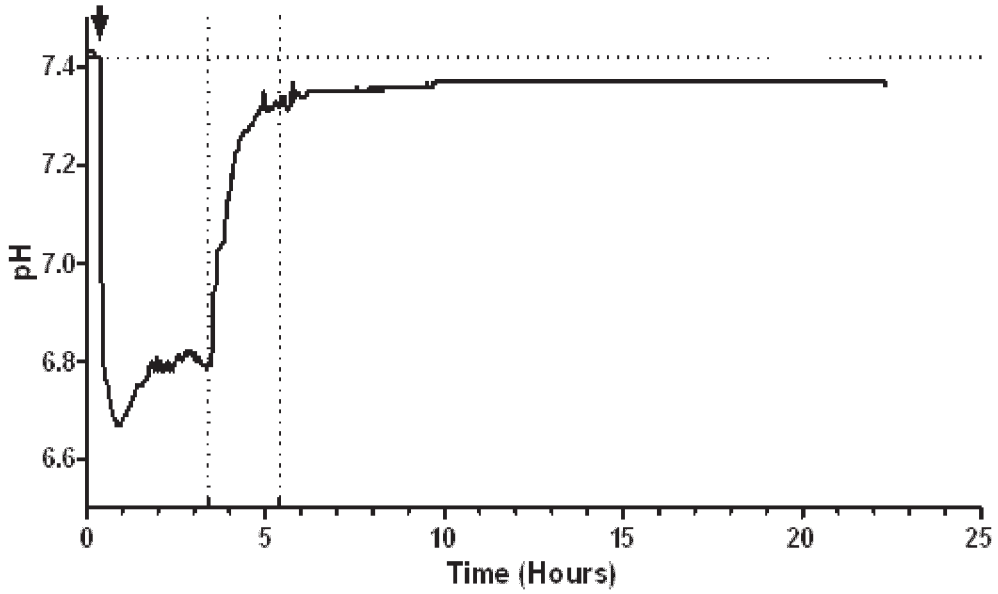


Figure 68

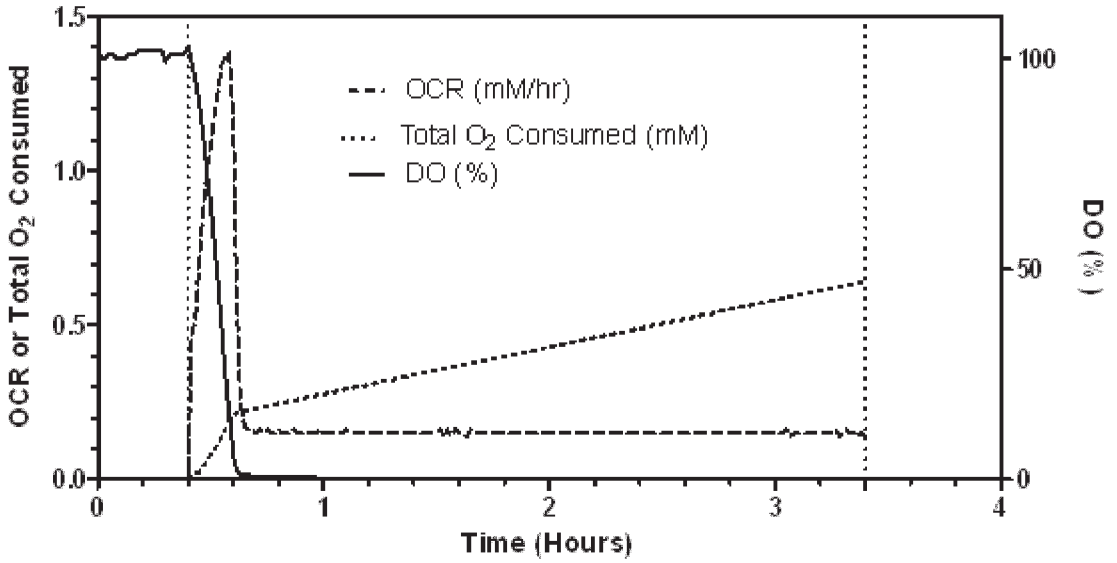


Figure 69

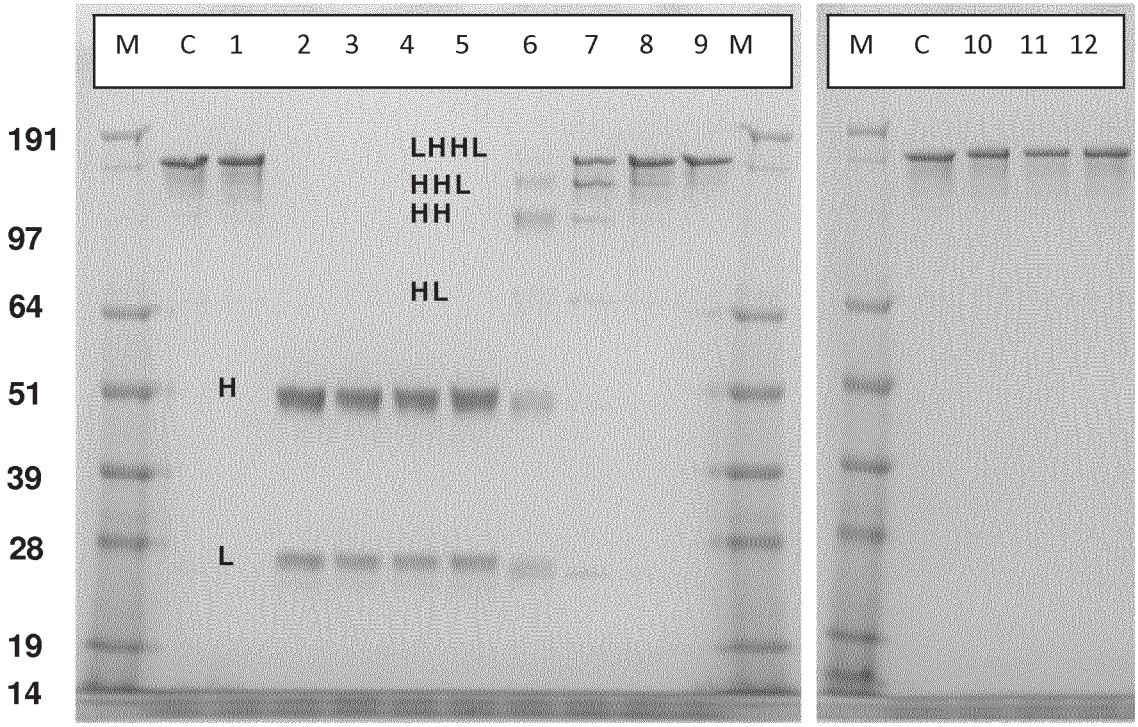

M C 1 2 3 4 5 6 7 8 9 M
M C 10 11 12
191
97
64
51
39
28
19
14
LHHL
HHL
HH
HL
H
L

Figure 70

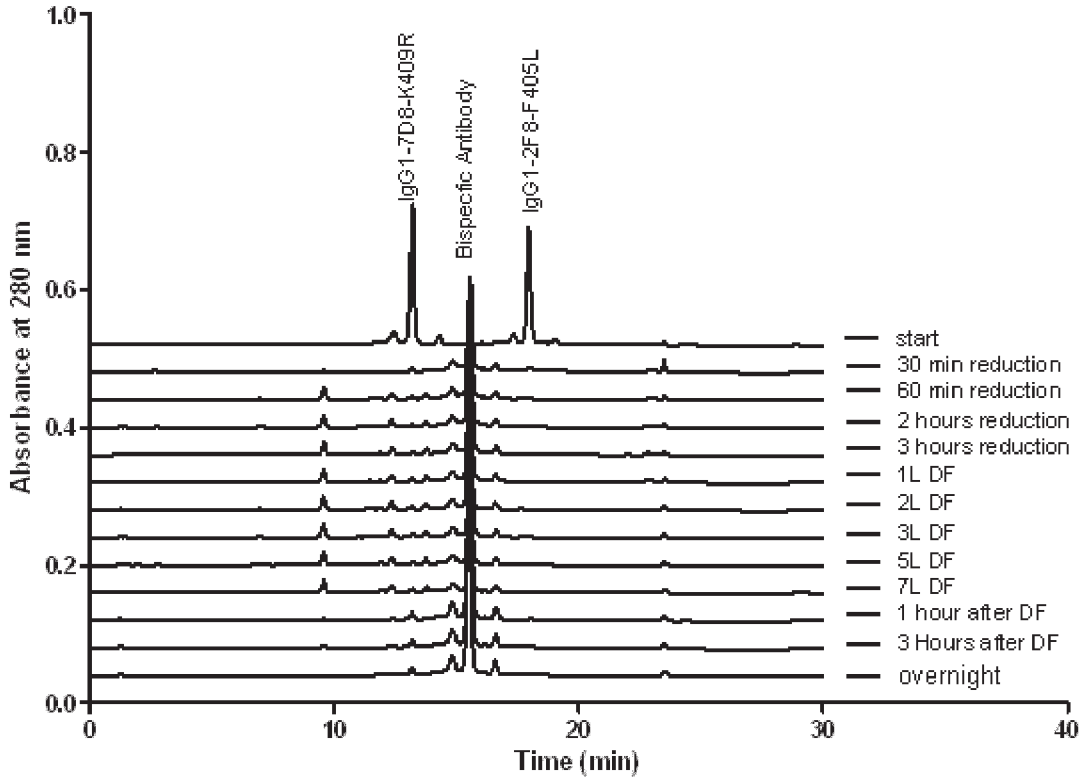


Figure 71

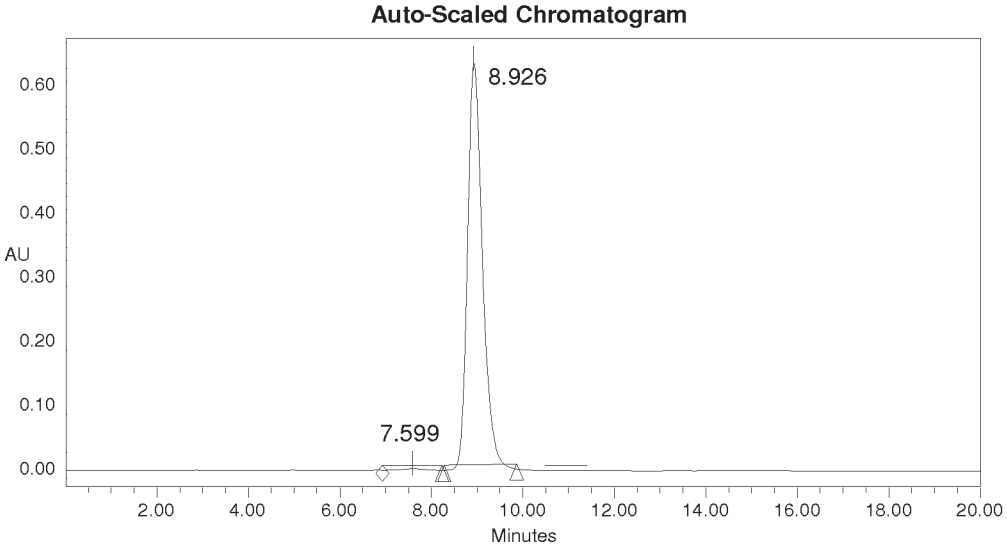


Figure 72

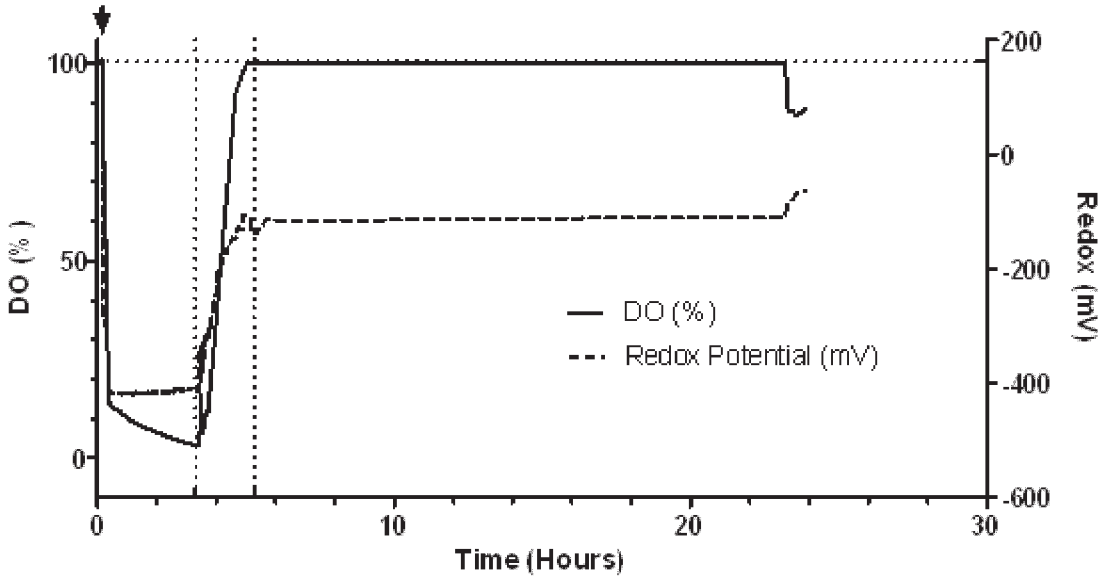


Figure 73

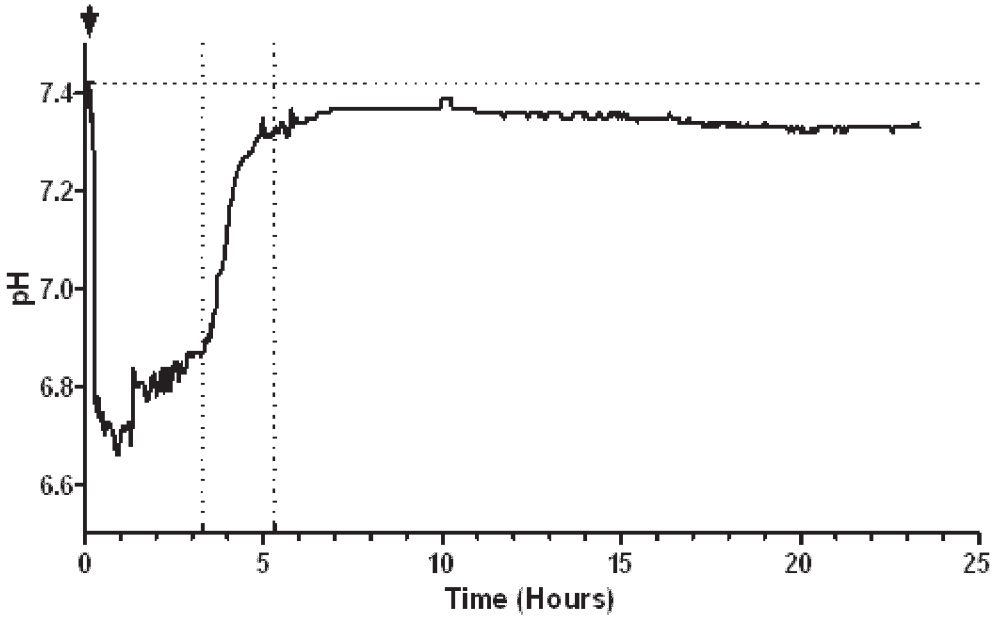


Figure 74

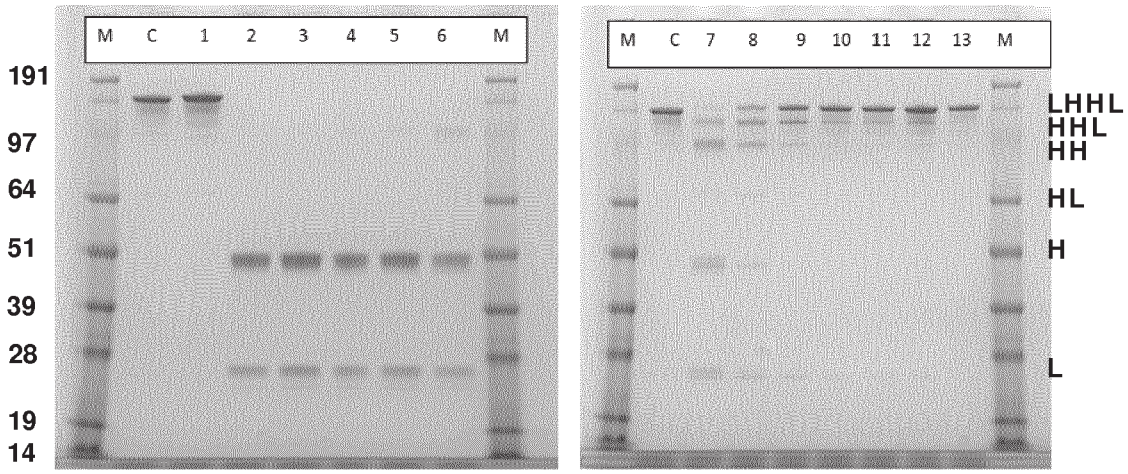


Figure 75

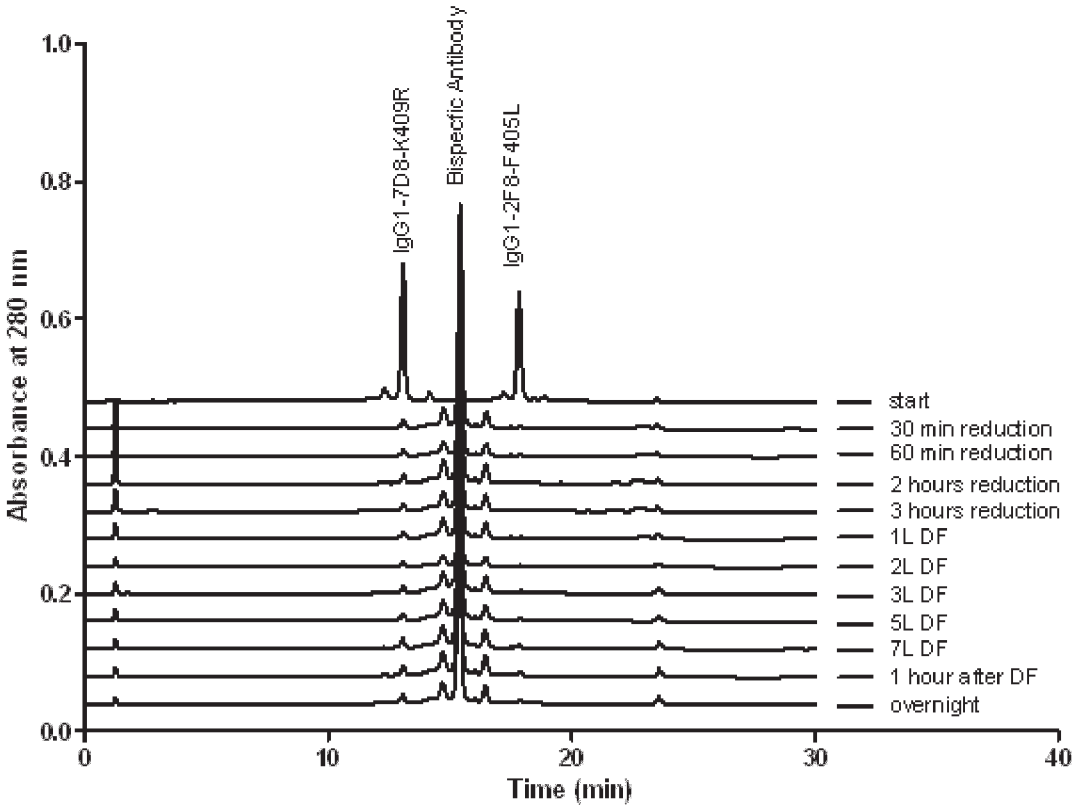


Figure 76

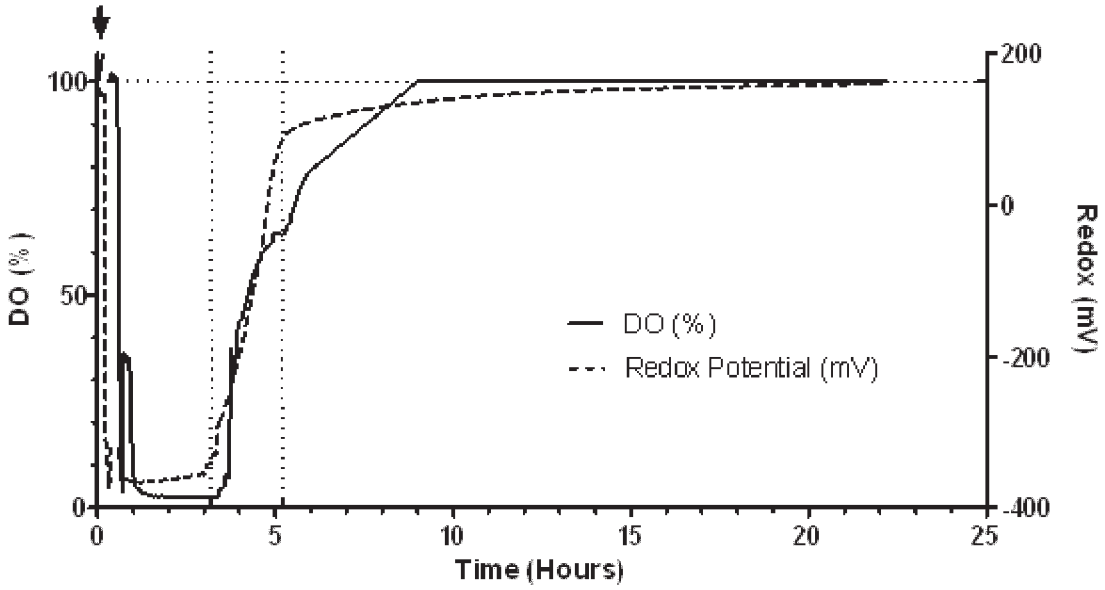


Figure 77

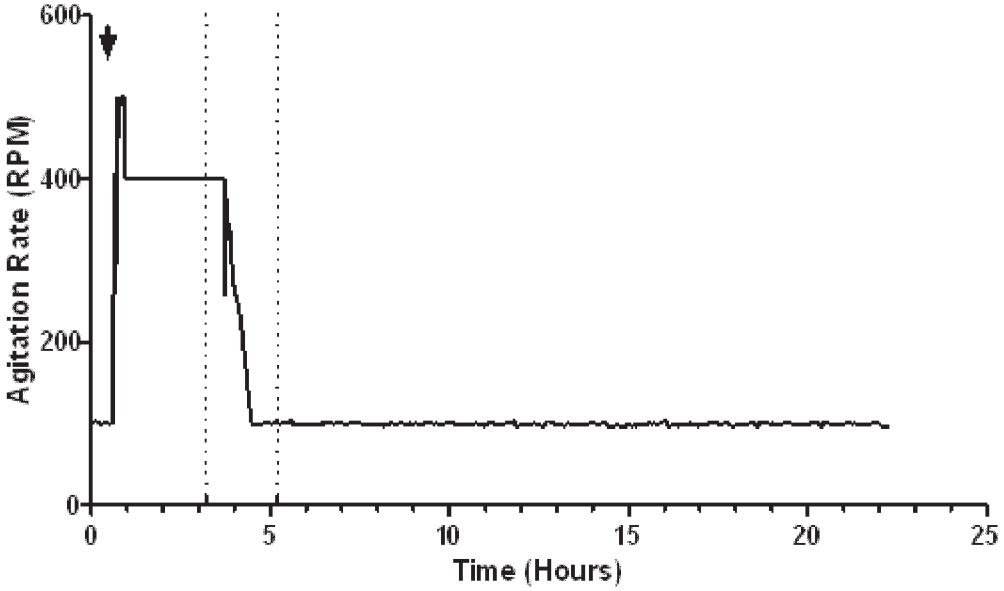


Figure 78

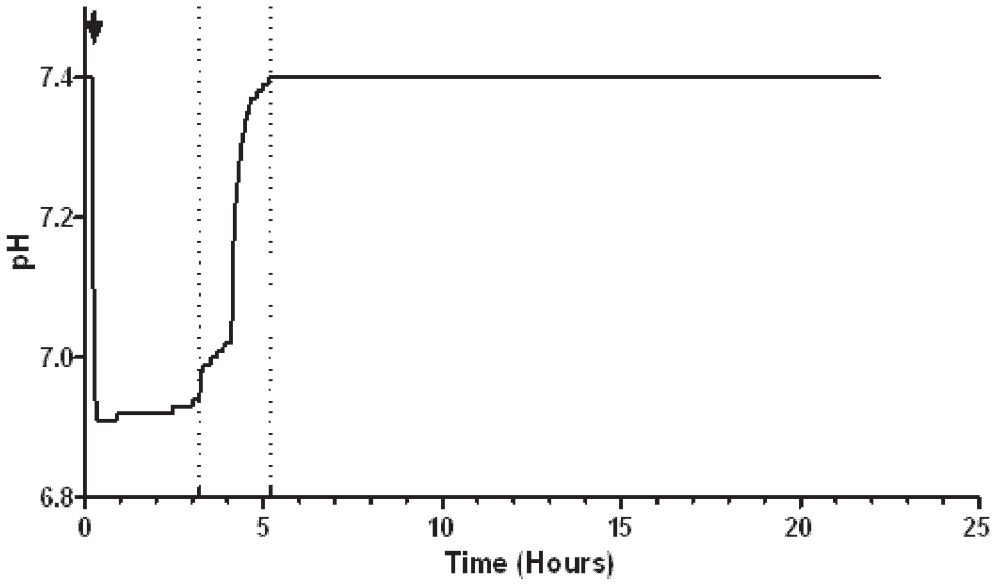


Figure 79

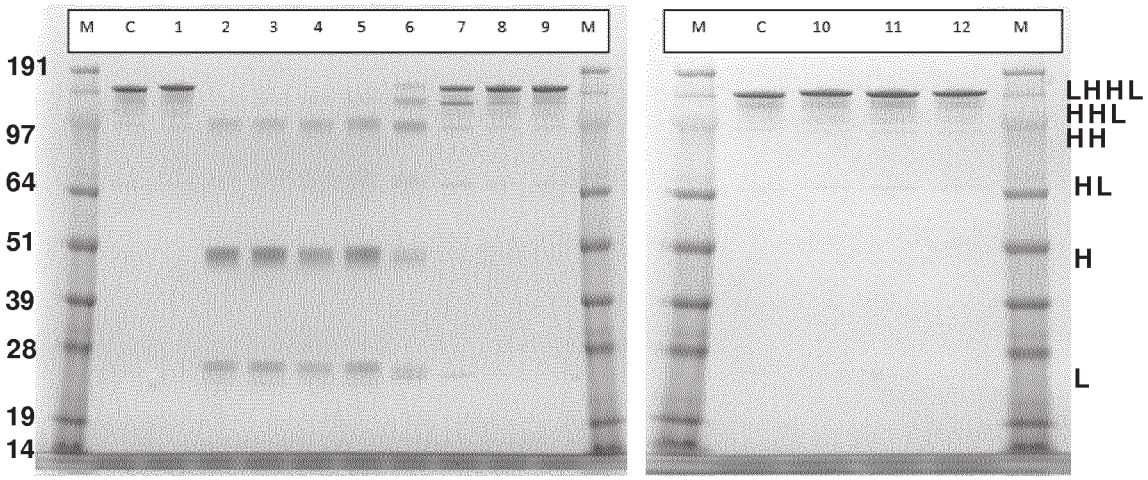


Figure 80

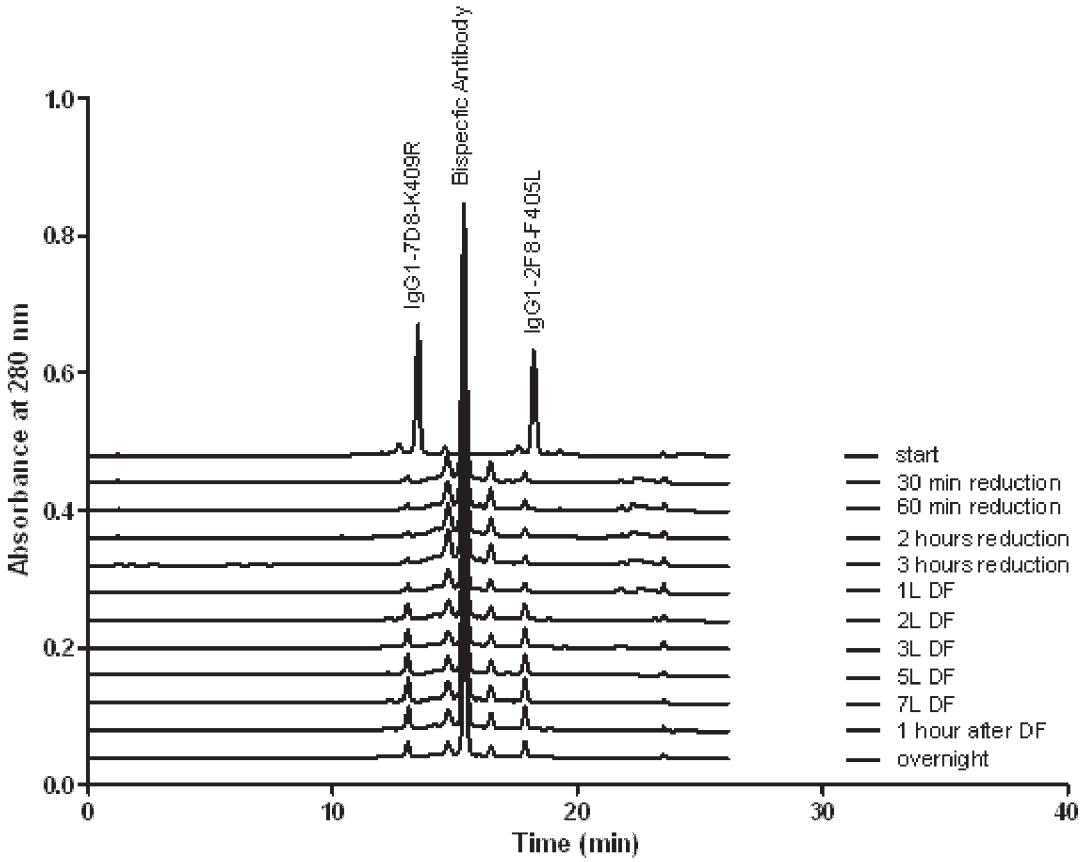


Figure 81

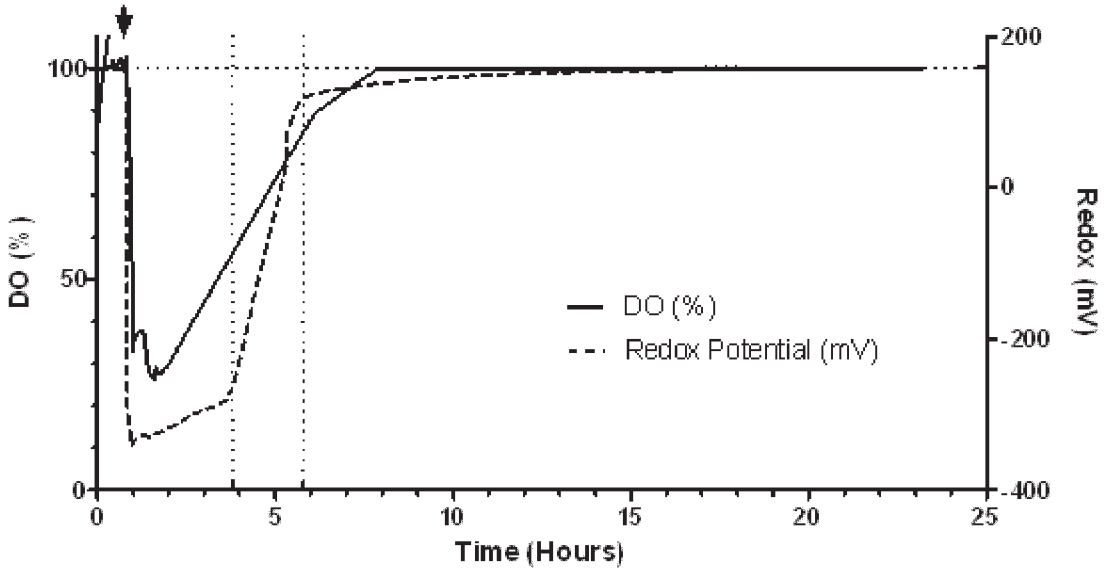


Figure 82

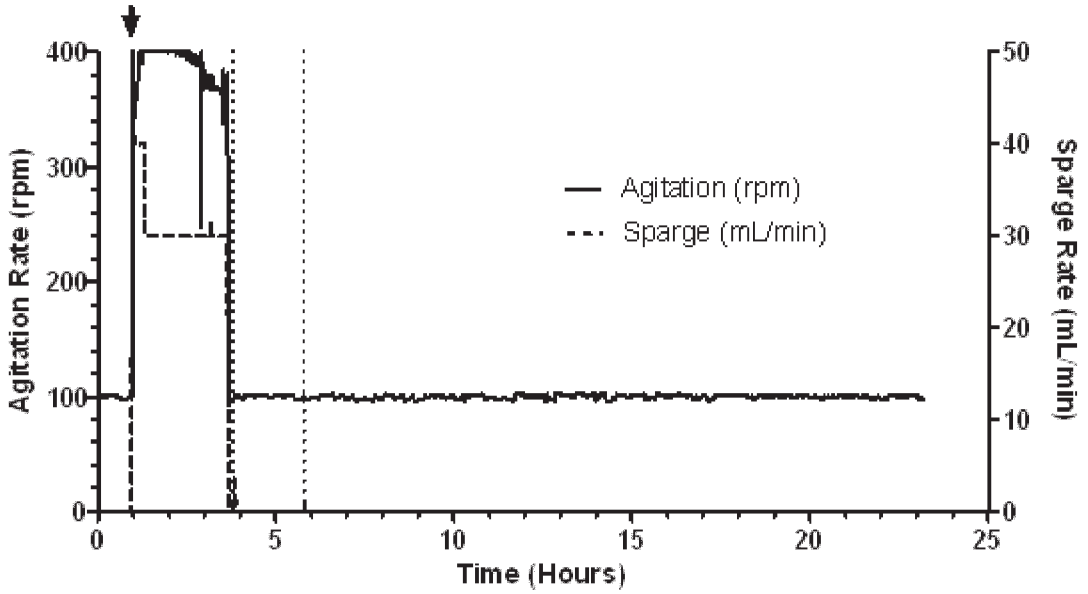


Figure 83

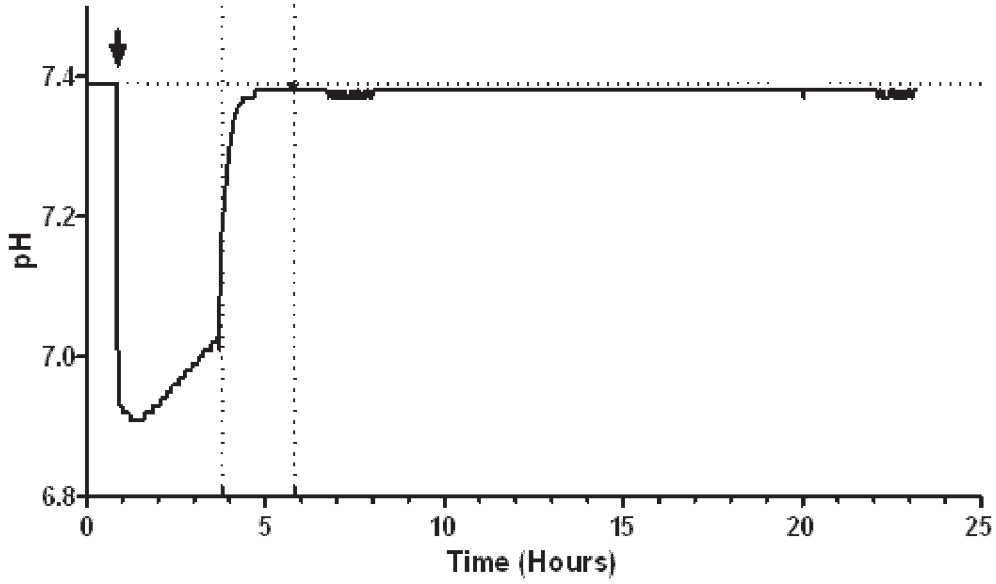


Figure 84

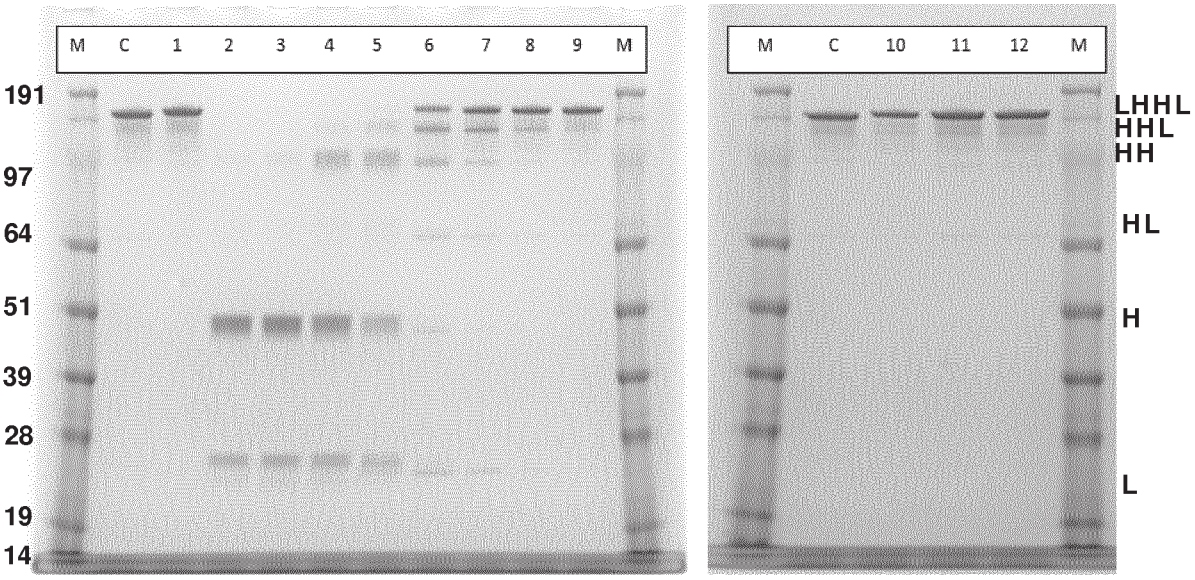


Figure 85

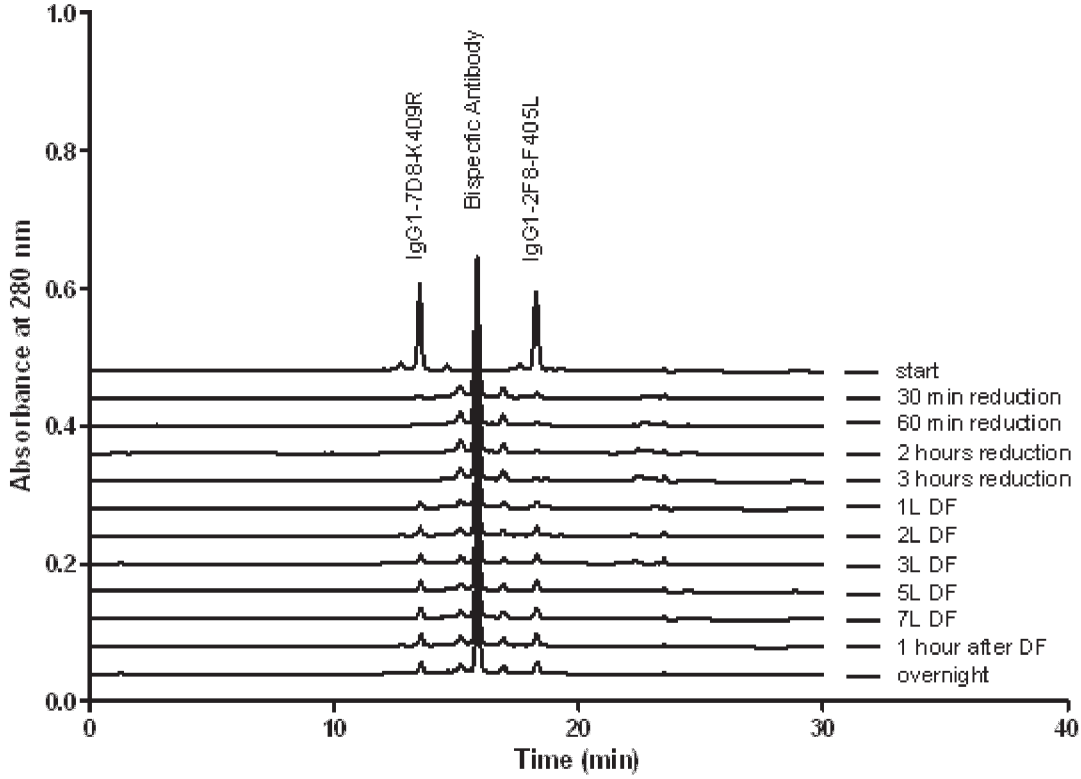


Figure 86

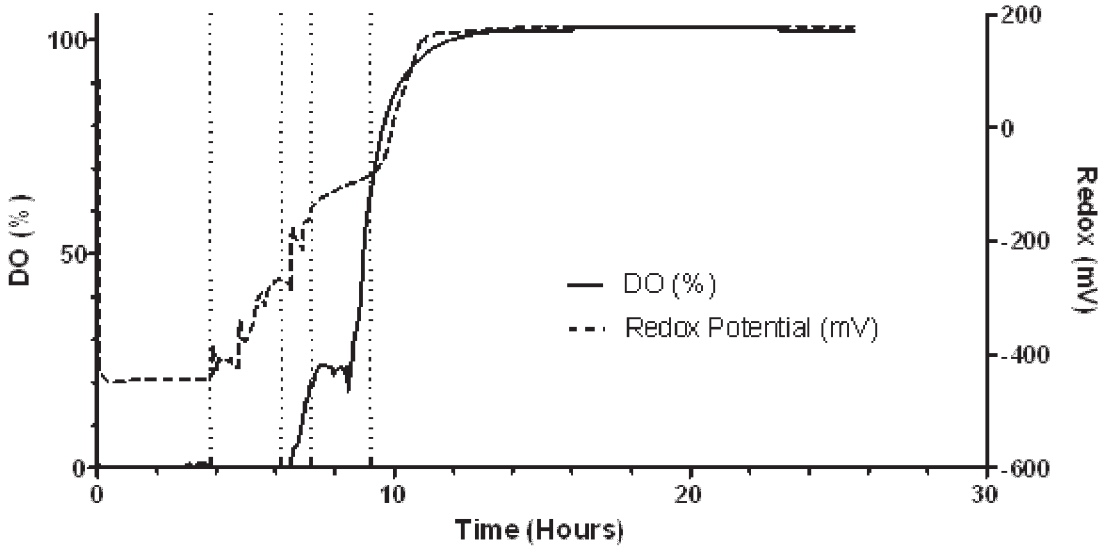


Figure 87

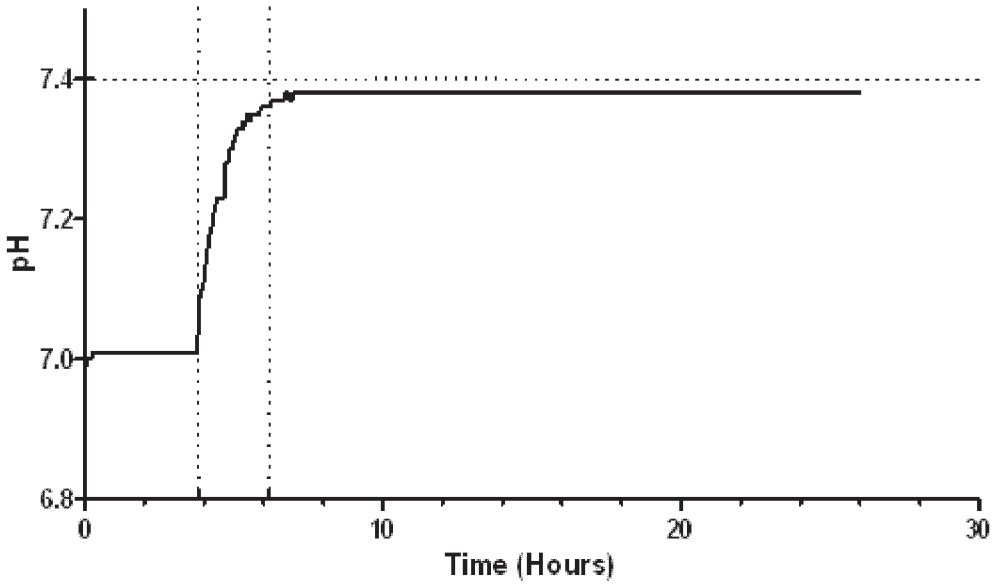


Figure 88

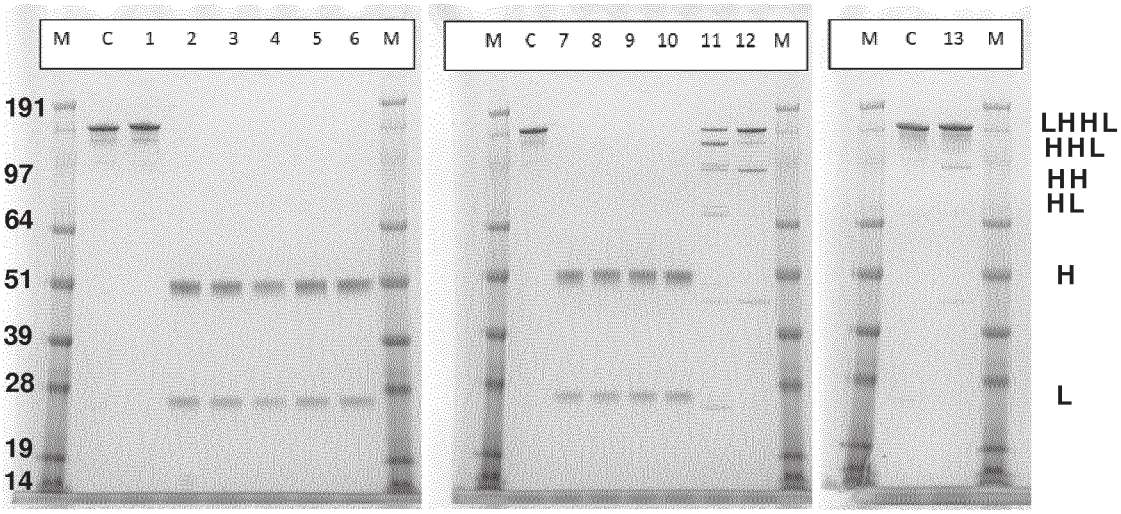


Figure 89

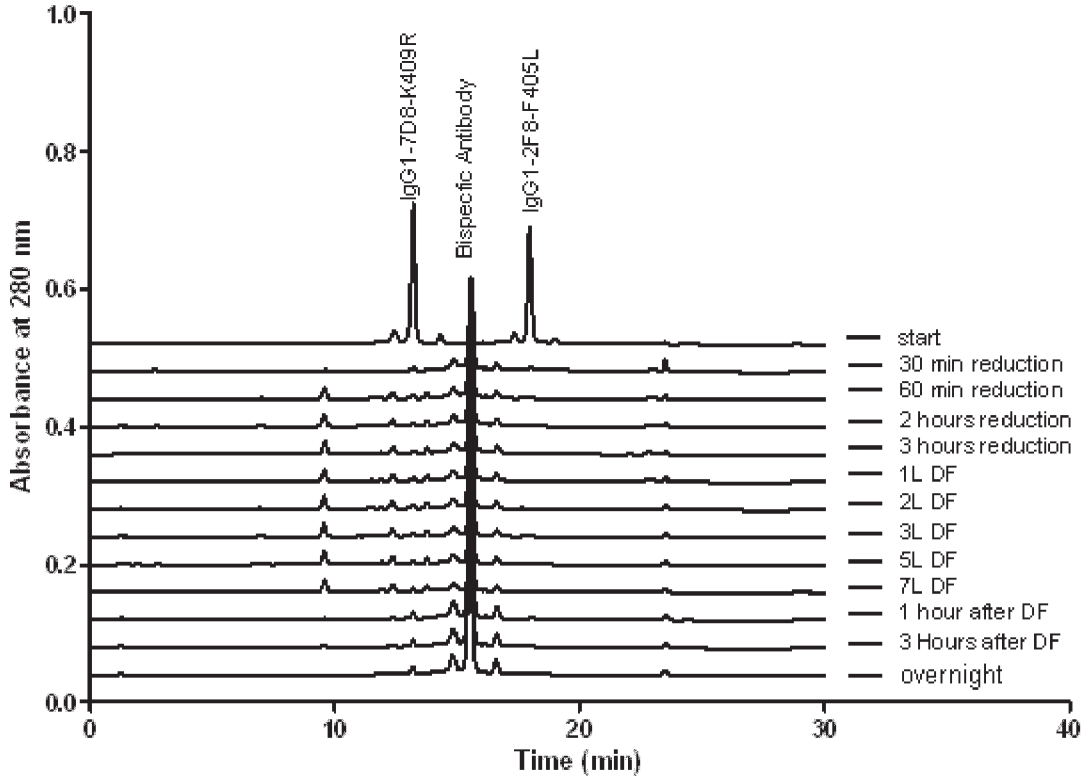


Figure 90

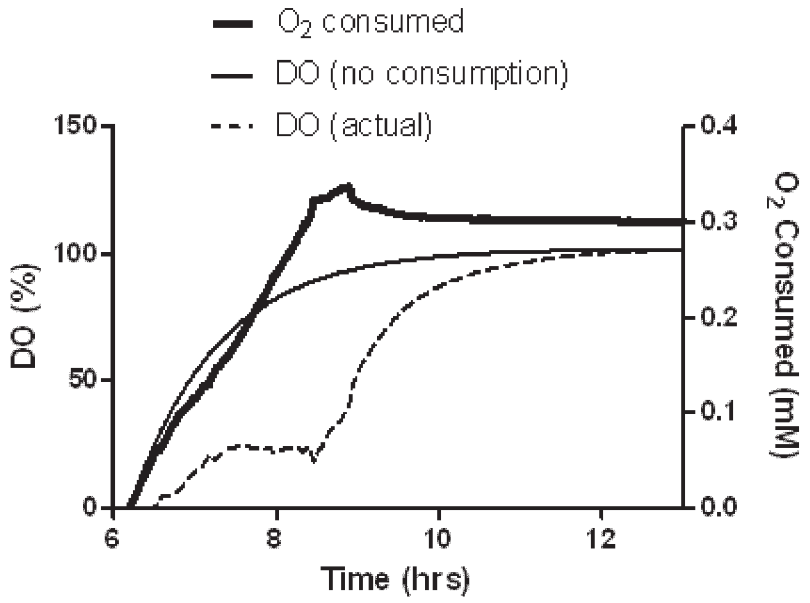


Figure 91

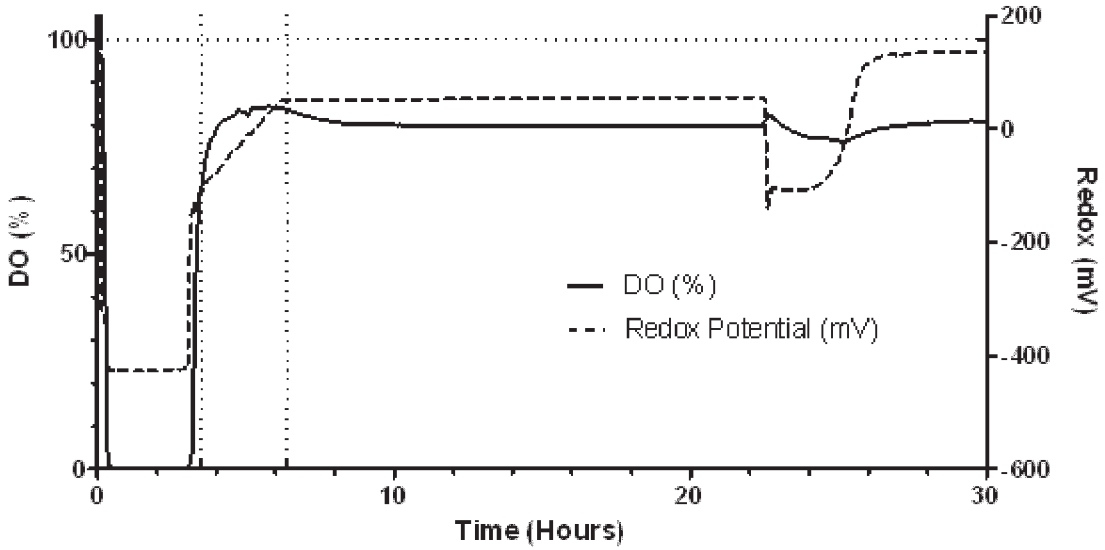


Figure 92

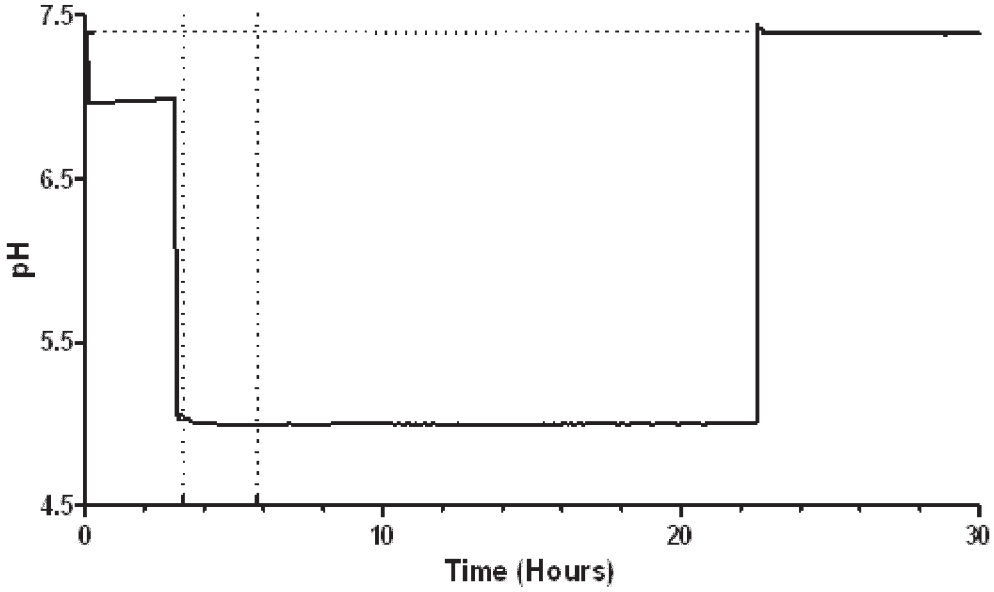


Figure 93

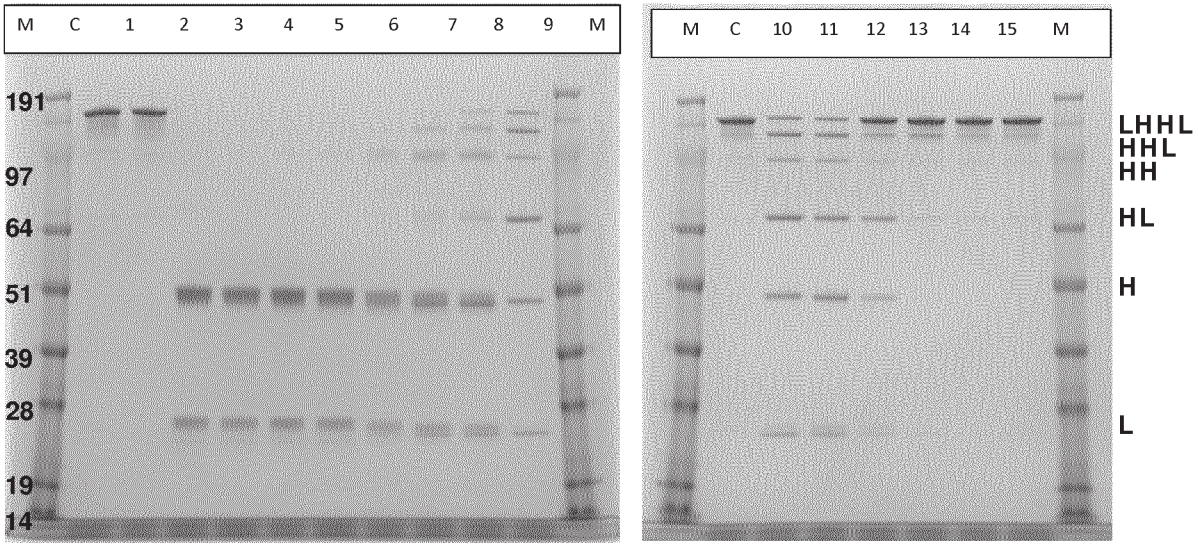


Figure 94

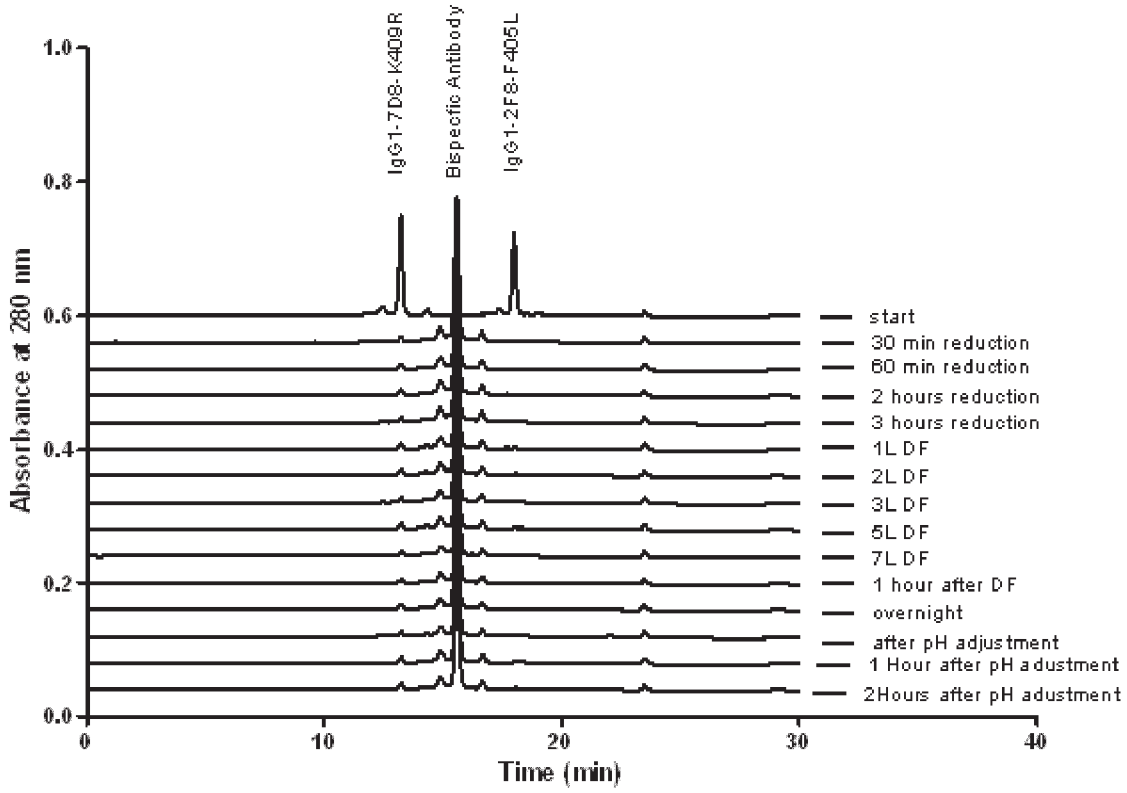


Figure 95

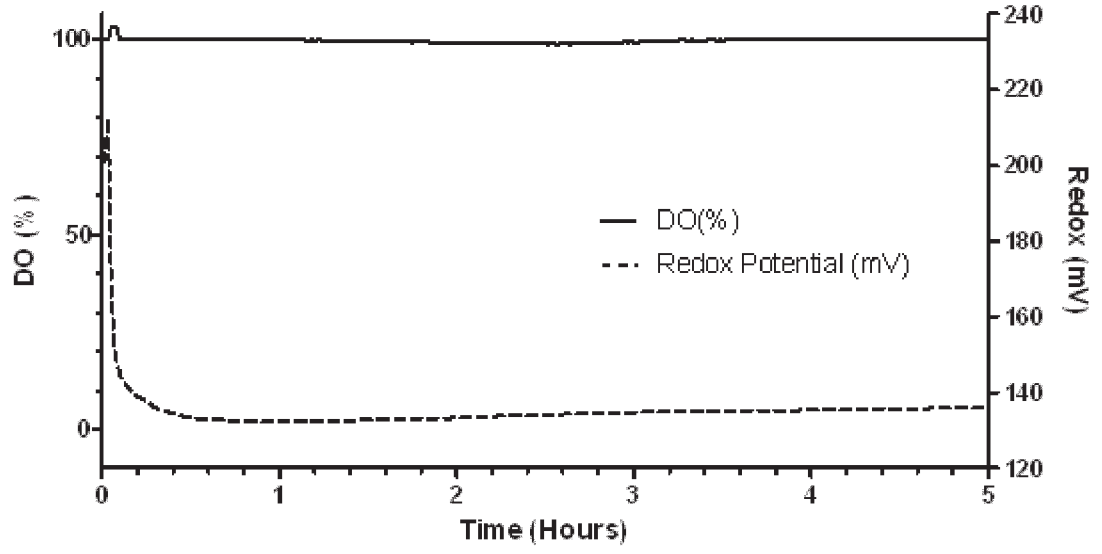


Figure 96

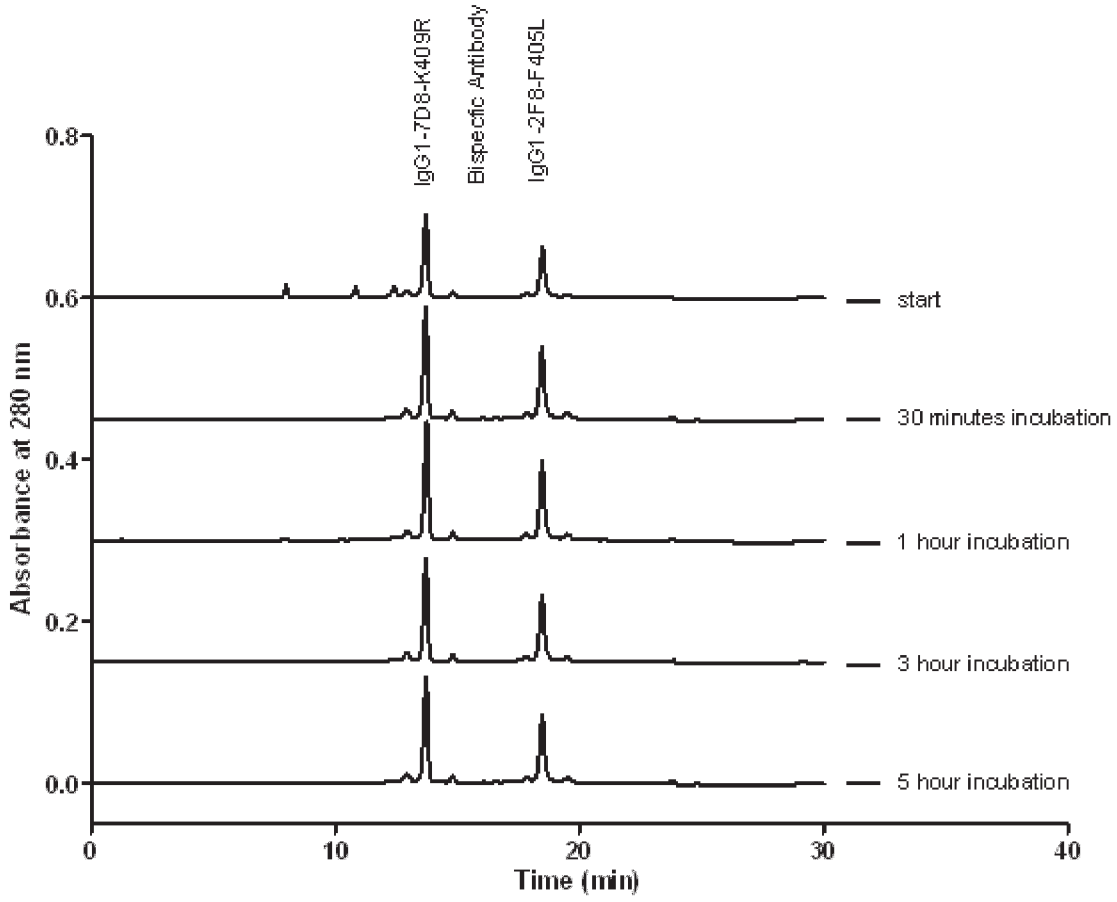

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
0.8
0.6
0.4
0.2
0.0
Absorbance at 280 nm
start
30 minutes incubation
1 hour incubation
3 hour incubation
5 hour incubation
0
10
20
30
40
Time (min)

Figure 97

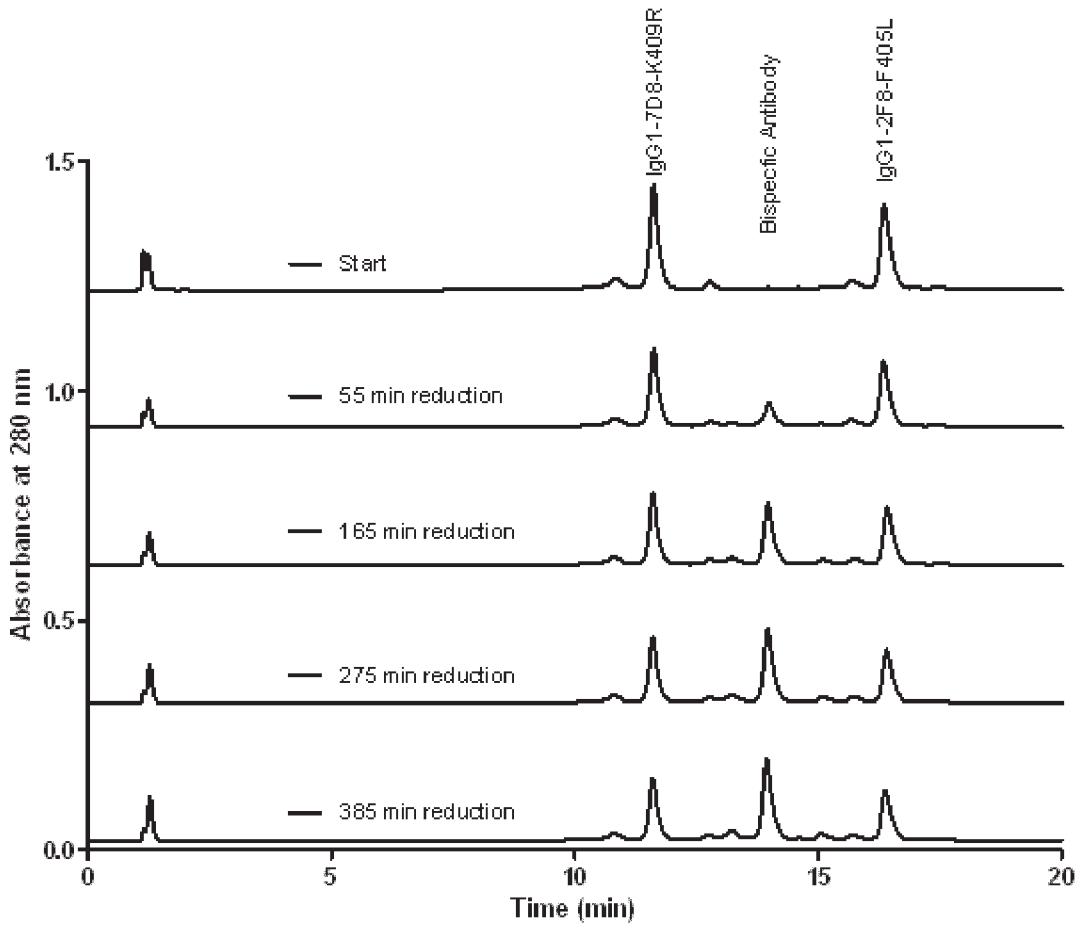

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
Start
55 min reduction
165 min reduction
275 min reduction
385 min reduction
Absorbance at 280 nm
1.5
1.0
0.5
0.0
0
5
10
15
20
Time (min)

Figure 98

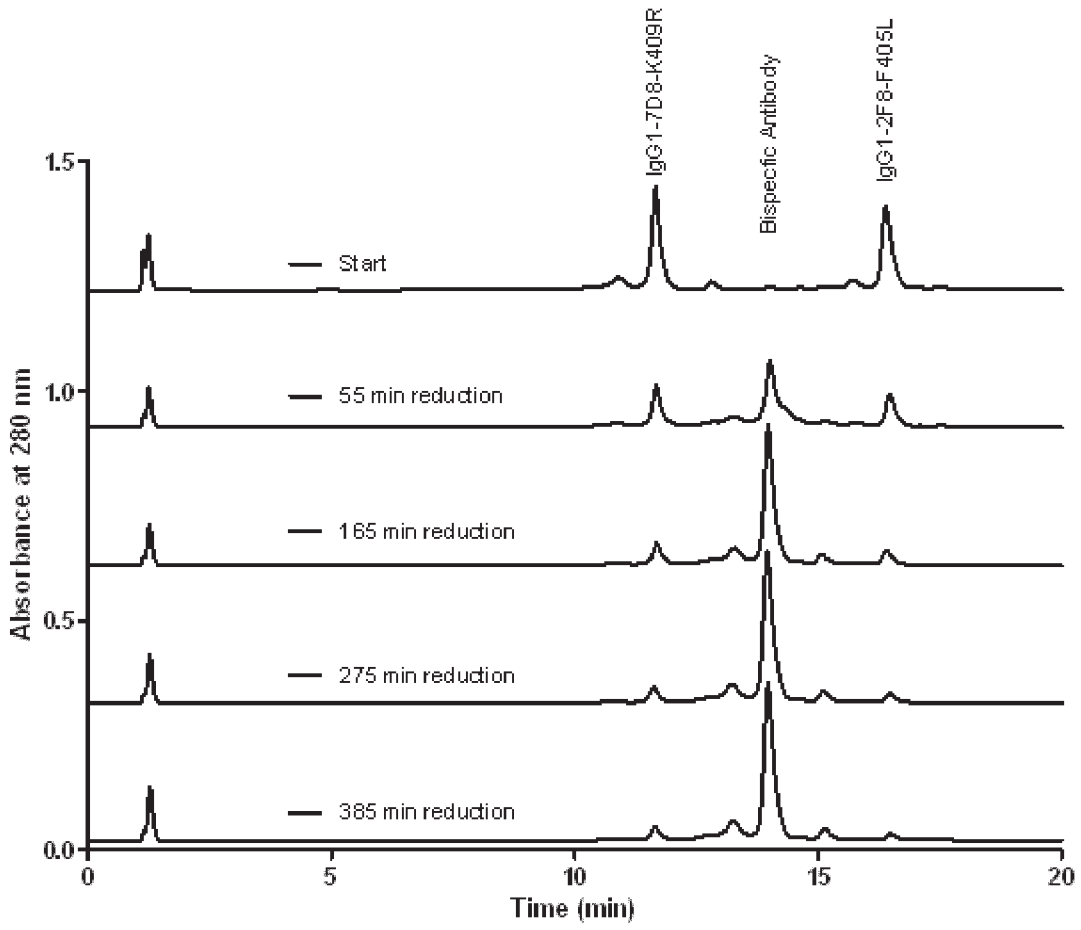

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
Start
55 min reduction
165 min reduction
275 min reduction
385 min reduction
Absorbance at 280 nm
1.5
1.0
0.5
0.0
0
5
10
15
20
Time (min)

Figure 99

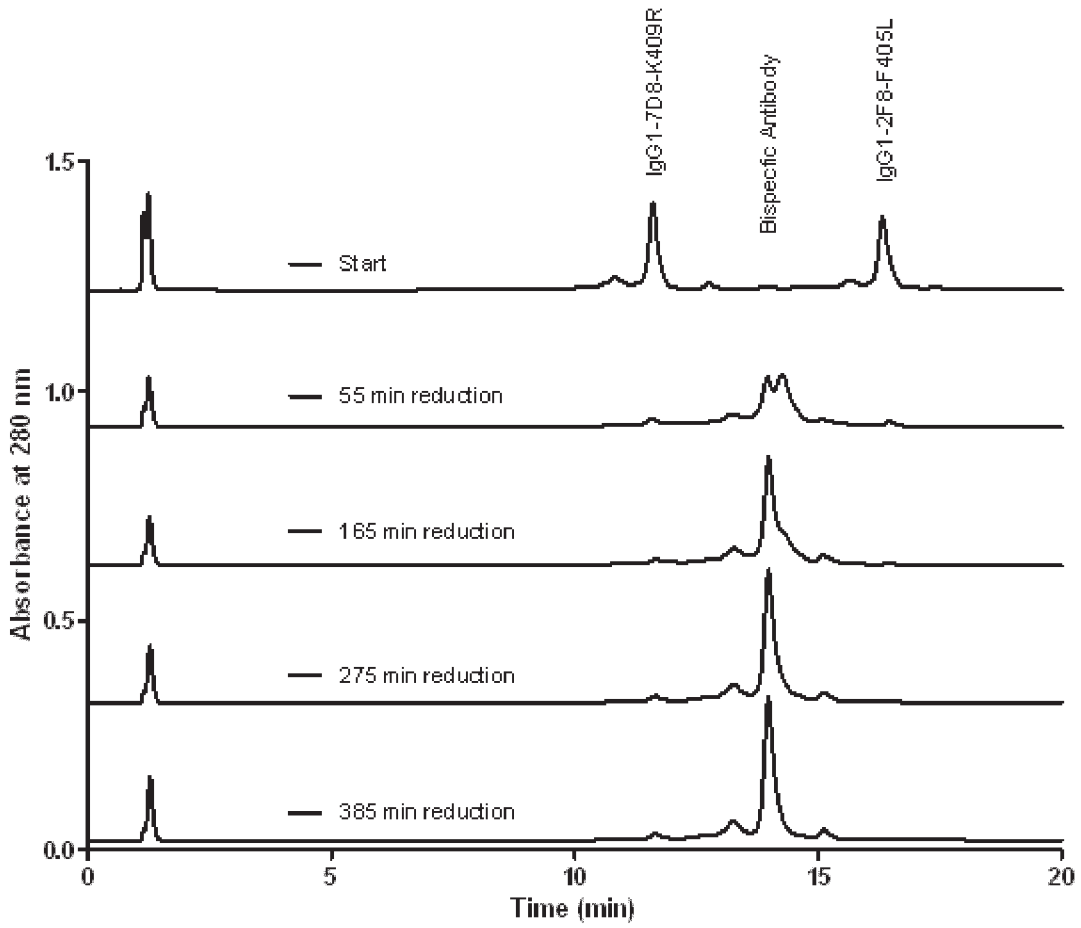

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
Start
55 min reduction
165 min reduction
275 min reduction
385 min reduction
Absorbance at 280 nm
1.5
1.0
0.5
0.0
0
5
10
15
20
Time (min)

Figure 100

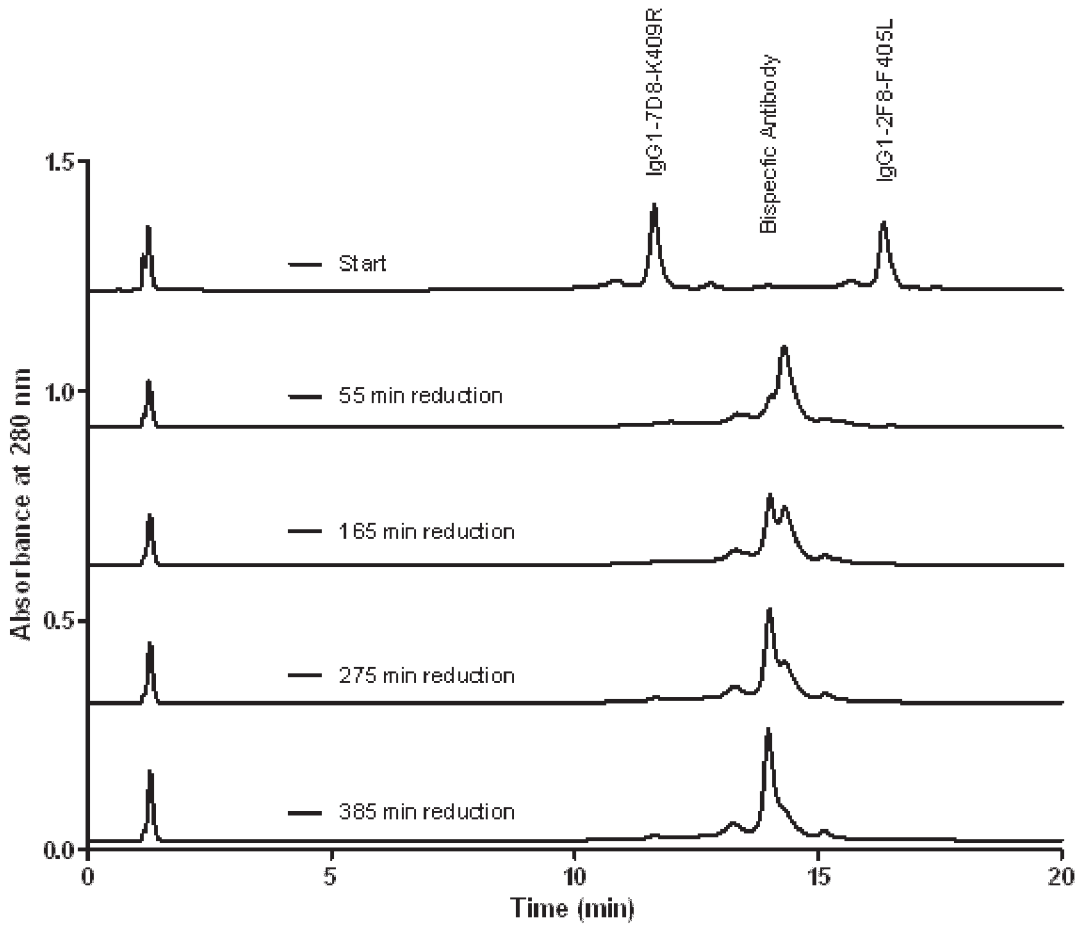

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
Start
55 min reduction
165 min reduction
275 min reduction
385 min reduction
Absorbance at 280 nm
1.5
1.0
0.5
0.0
0
5
10
15
20
Time (min)

Figure 101

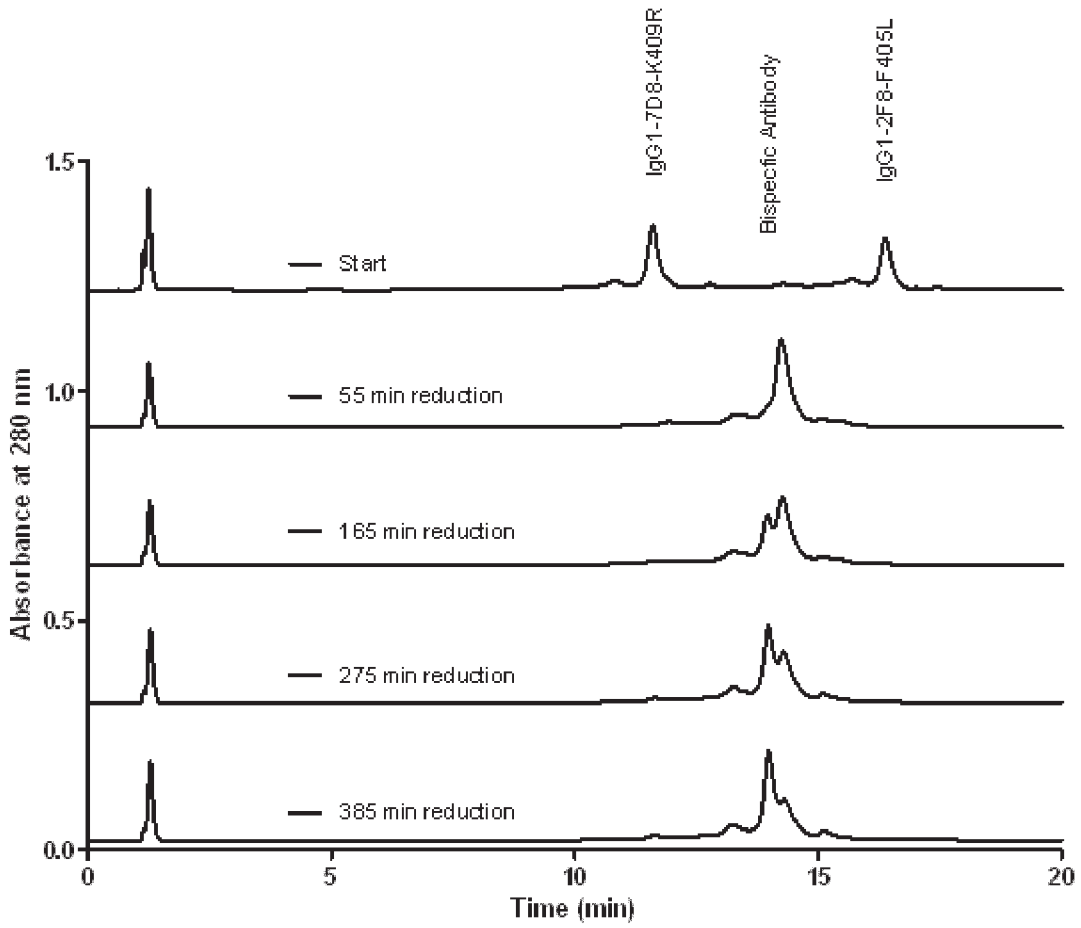

IgG1-7D8-K409R
Bispecific Antibody
IgG1-2F8-F405L
Start
55 min reduction
165 min reduction
275 min reduction
385 min reduction
Absorbance at 280 nm
1.5
1.0
0.5
0.0
0
5
10
15
20
Time (min)

Figure 102

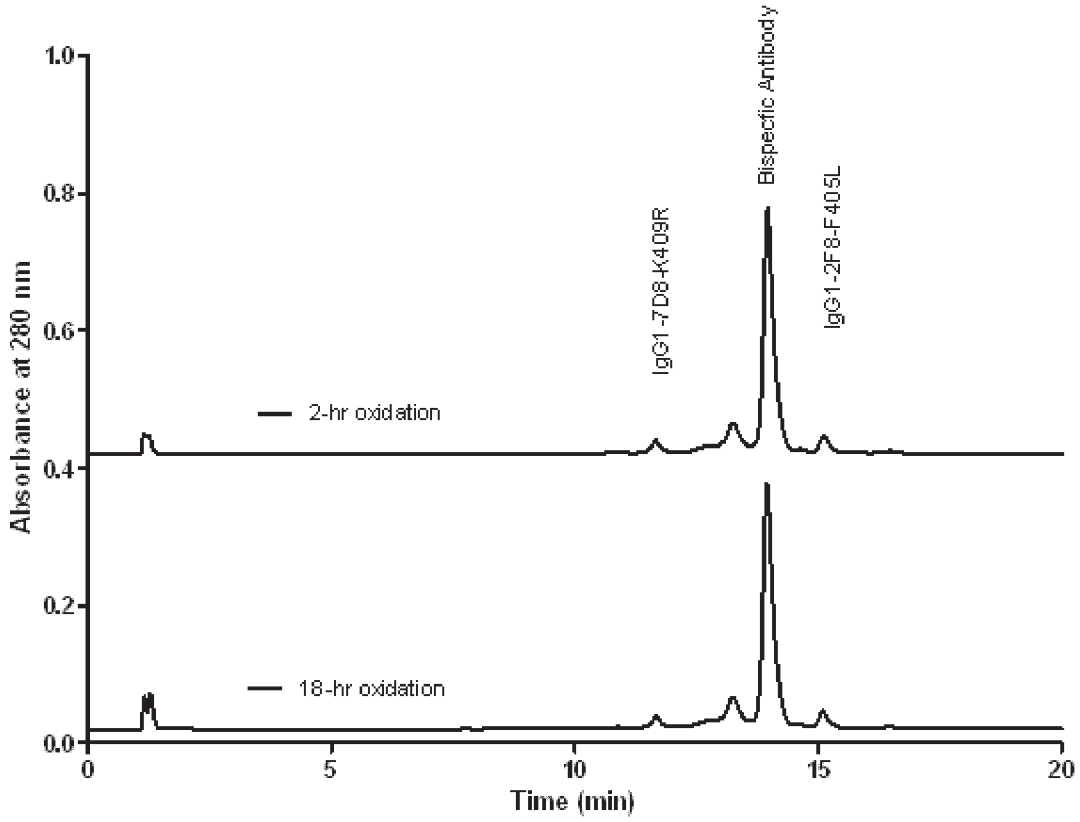

Bispecific Antibody
IgG1-7D8-K409R
IgG1-2F8-F405L
2-hr oxidation
18-hr oxidation
Absorbance at 280 nm
Time (min)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20

Figure 103

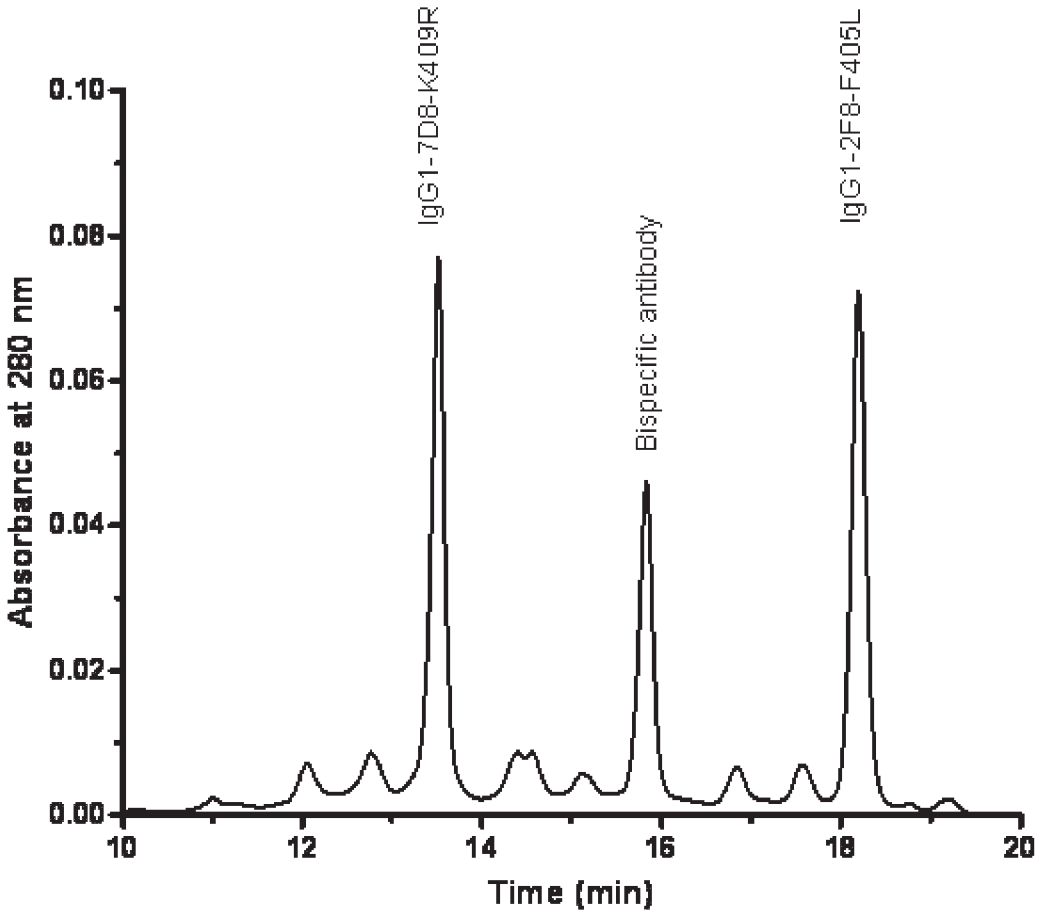

IgG1-7D8-K409R
Bispecific antibody
IgG1-2F8-F405L
Absorbance at 280 nm
0.10
0.08
0.06
0.04
0.02
0.00
10
12
14
16
18
20
Time (min)

Figure 104

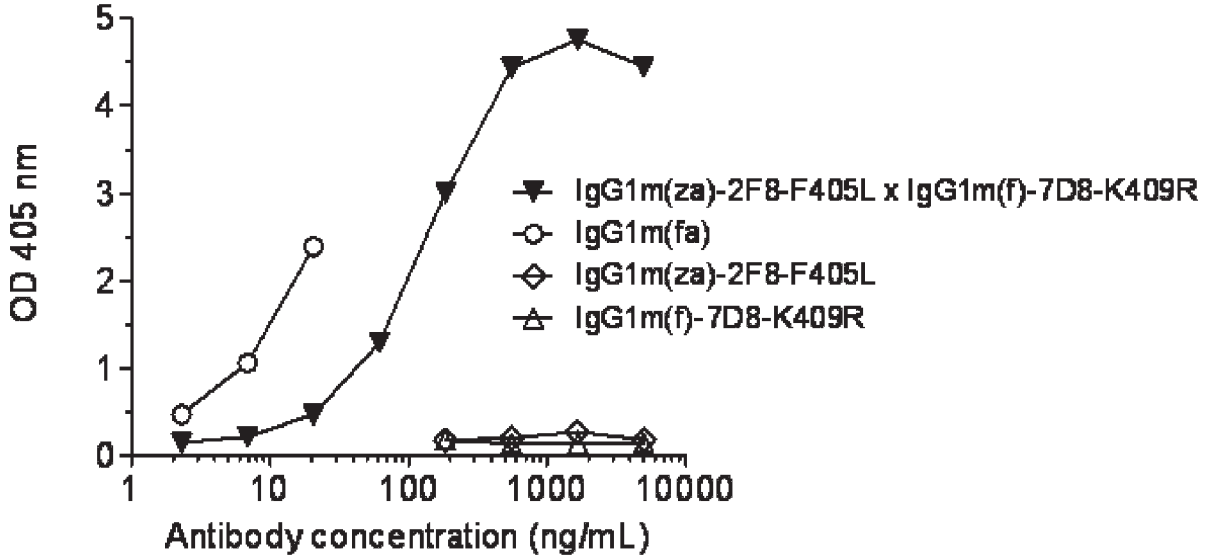


Figure 105

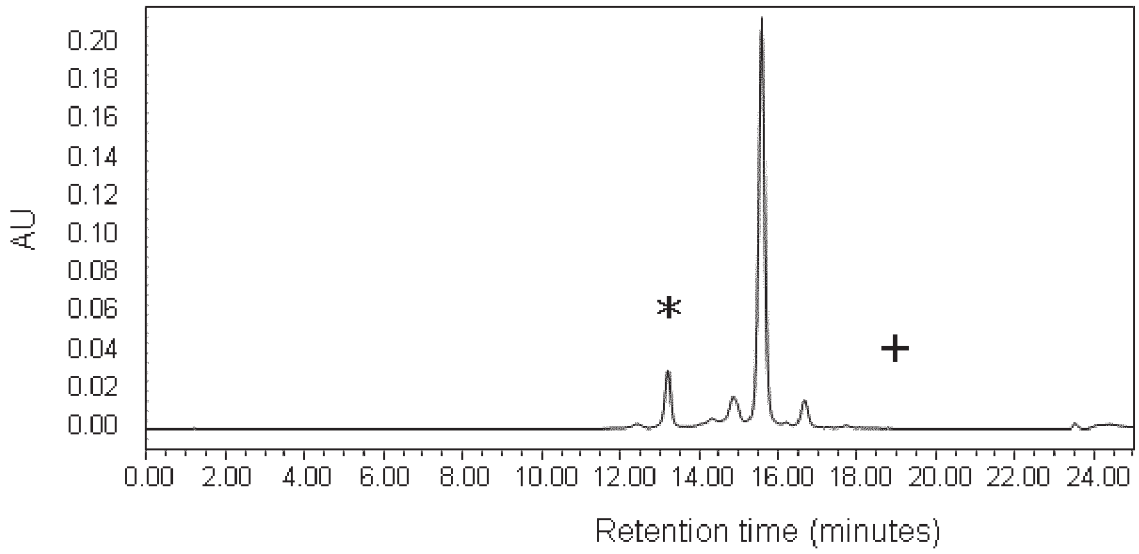


Figure 106

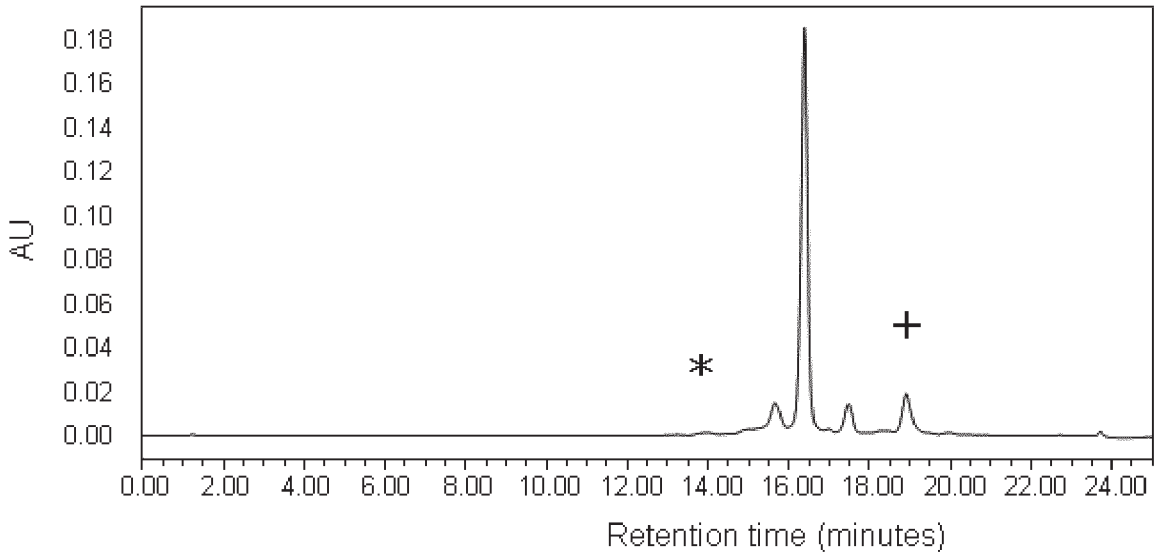


Figure 107

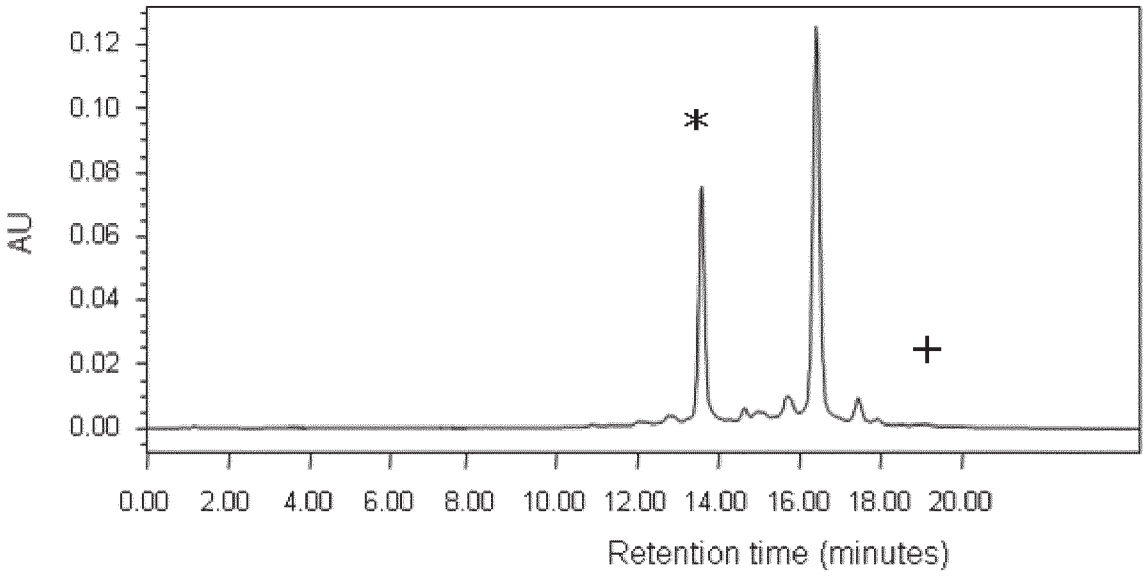


Figure 108

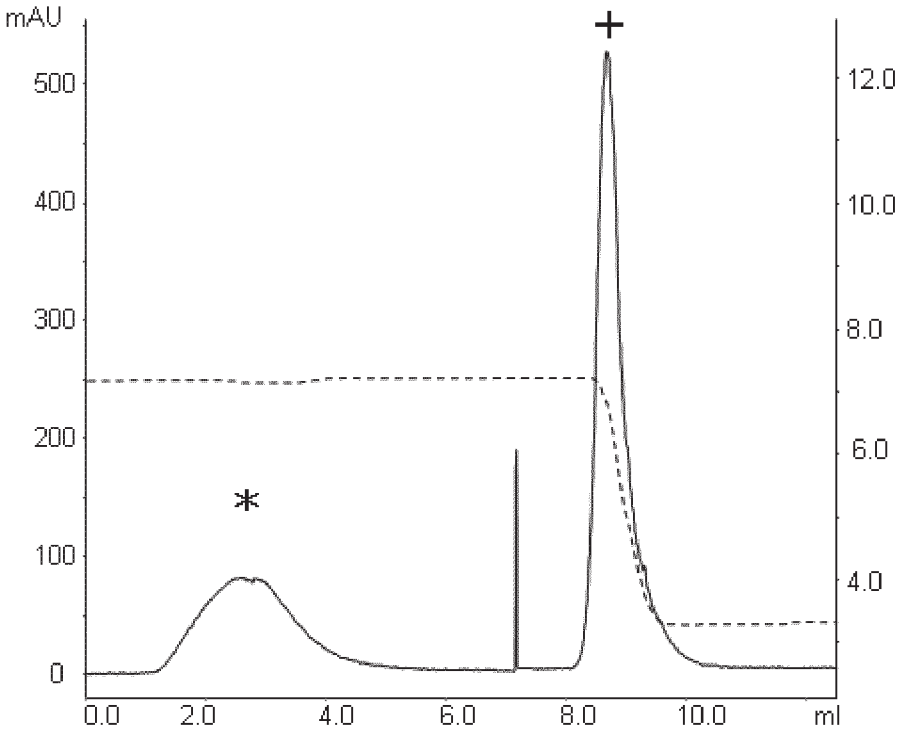


Figure 109

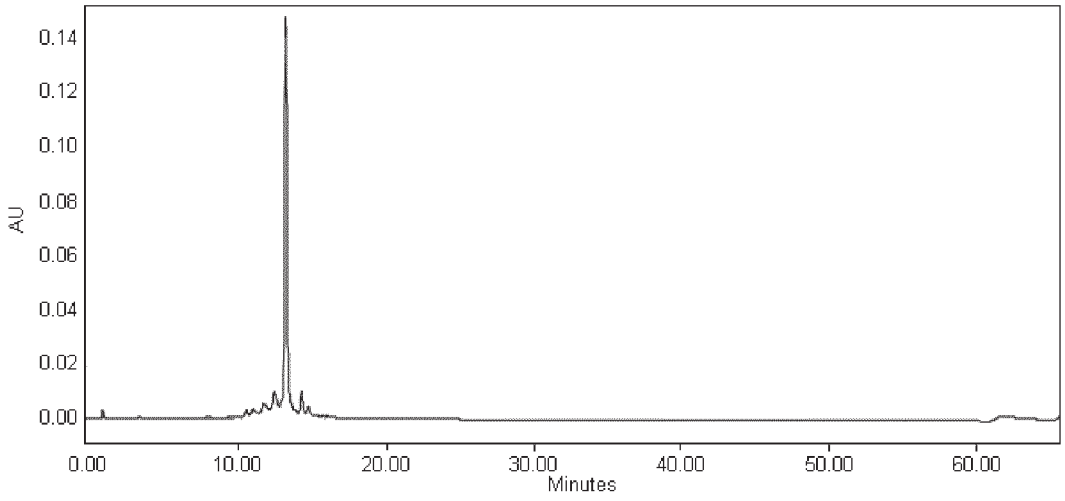


Figure 110

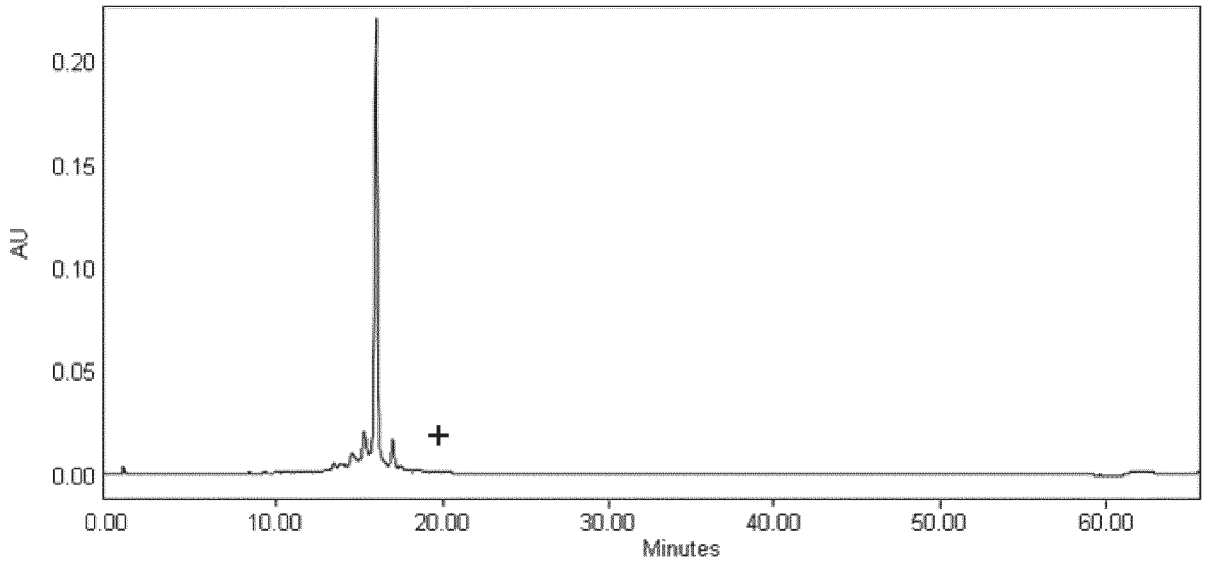


Figure 111

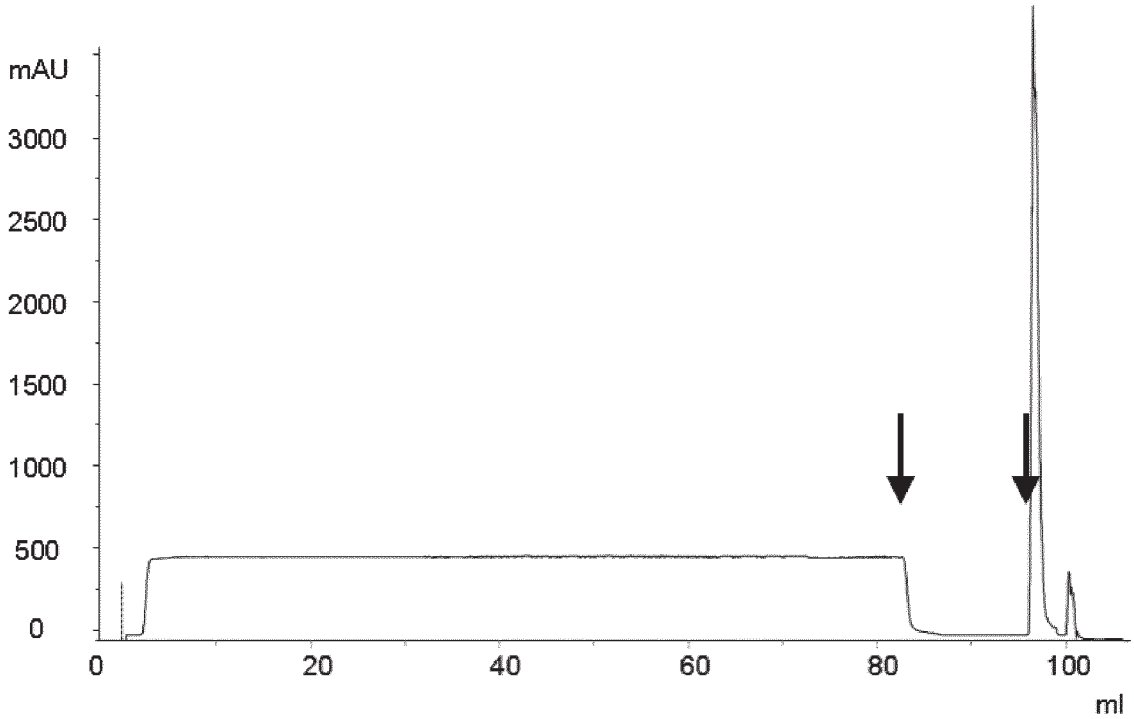


Figure 112

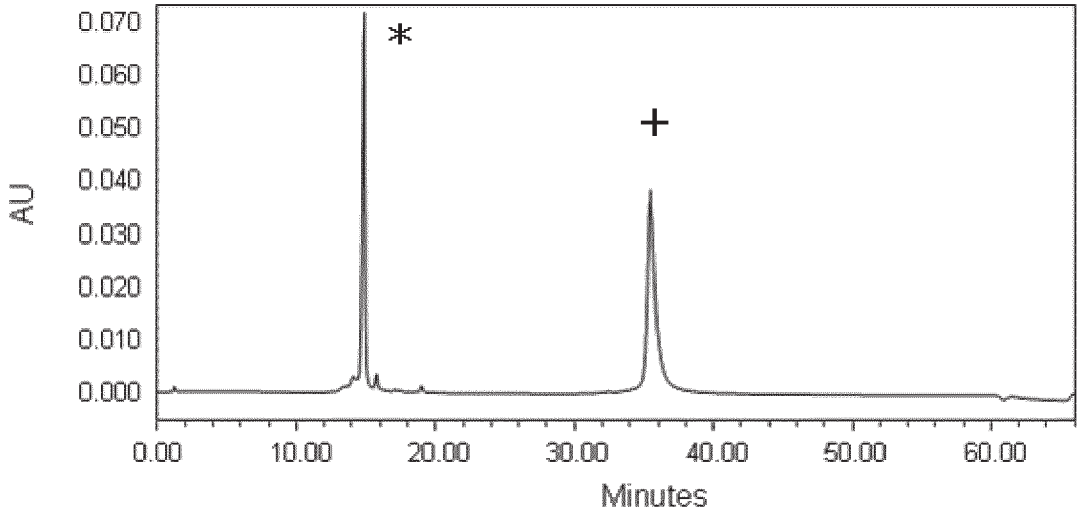


Figure 113

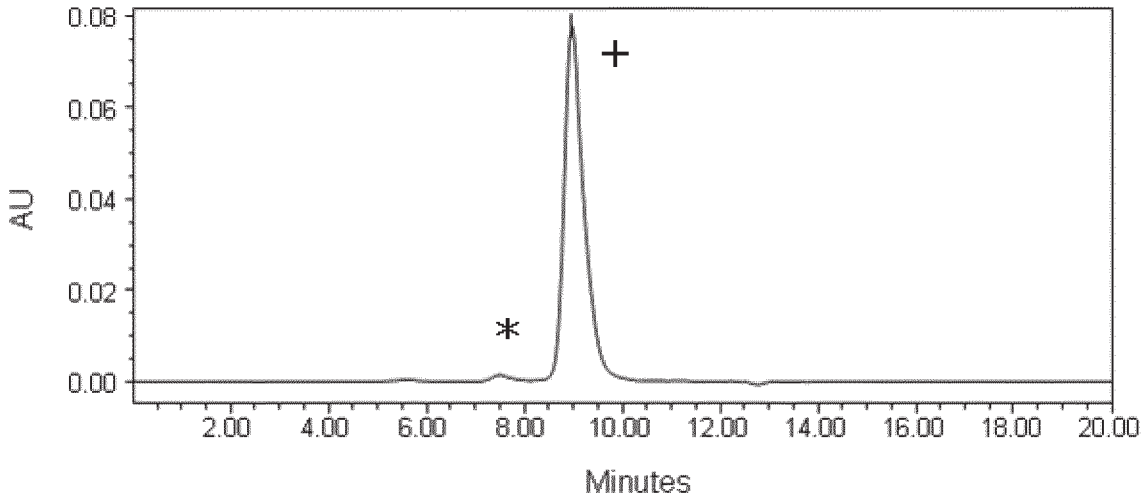


Figure 114

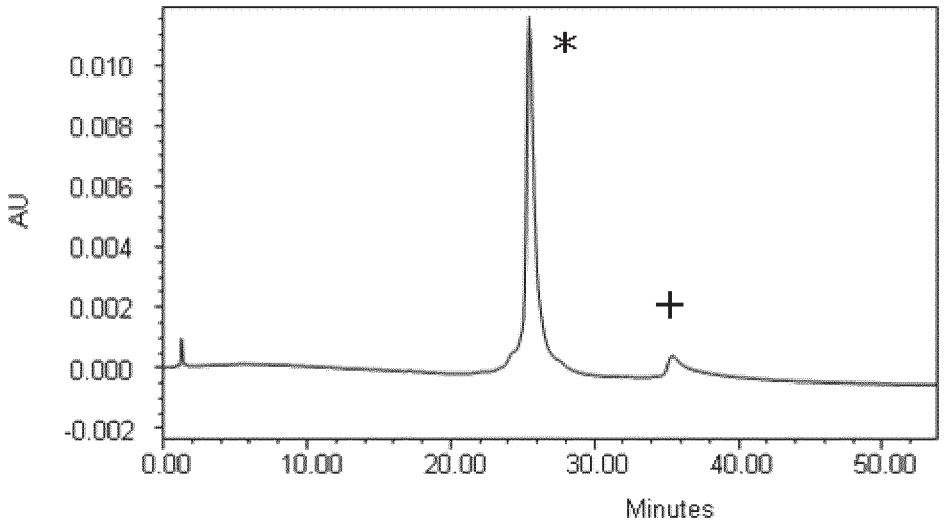

0.010
0.008
0.006
0.004
0.002
0.000
-0.002
AU
0.00
10.00
20.00
30.00
40.00
50.00
Minutes
*
+

Figure 115

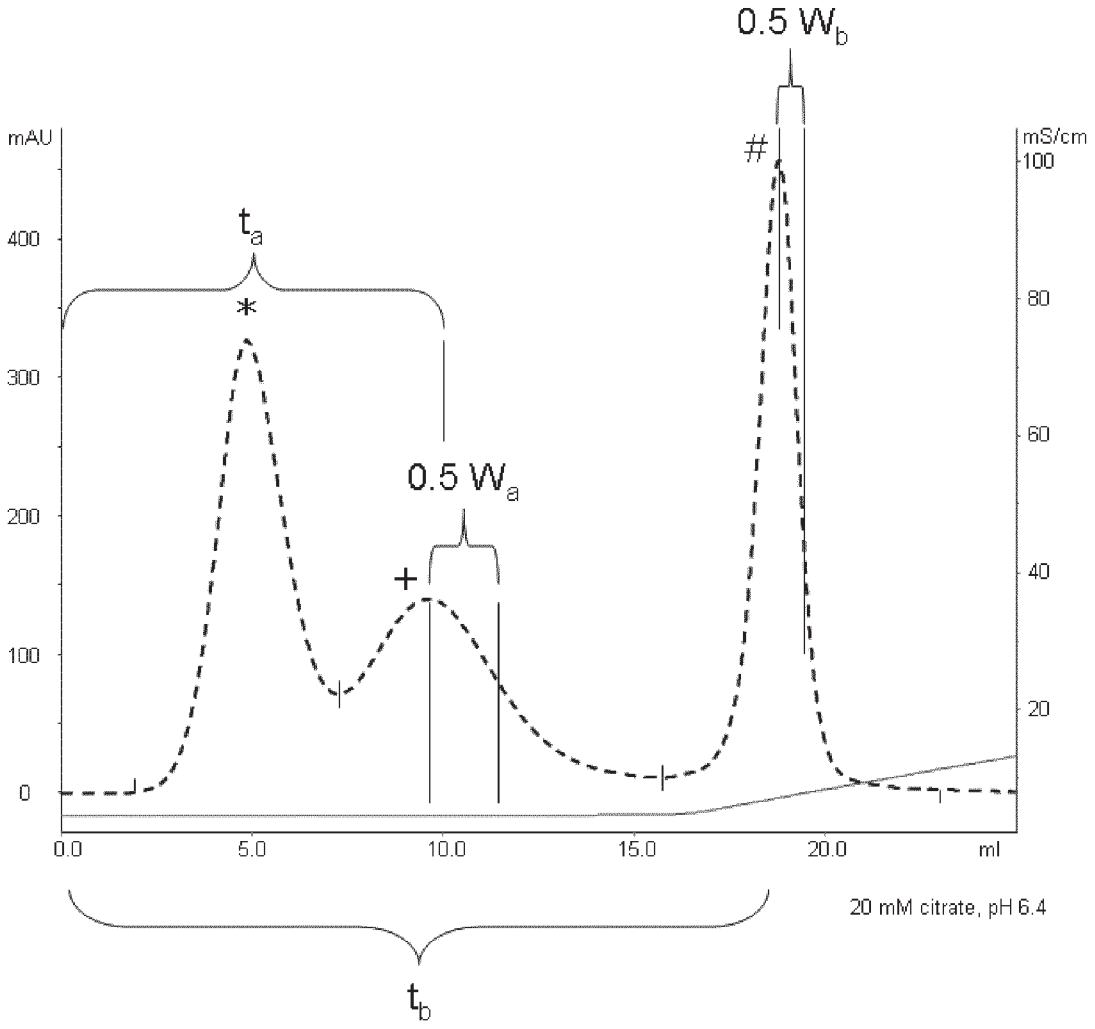

0.5 Wb
mAU
mS/cm
#
100
ta
400
*
80
300
60
0.5 Wa
200
+
40
100
20
0
0.0
5.0
10.0
15.0
20.0
ml
20 mM citrate, pH 6.4
tb

Figure 116

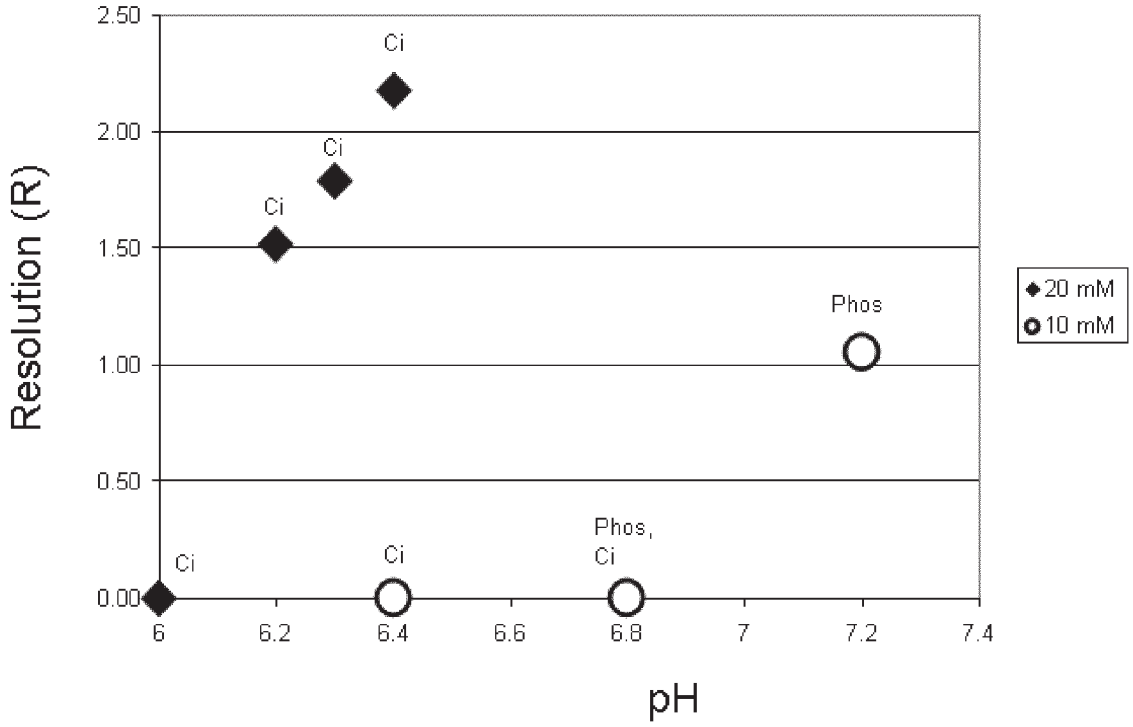

2.50
2.00
1.50
1.00
0.50
0.00
Resolution (R)
6
6.2
6.4
6.6
6.8
7
7.2
7.4
pH
Ci
Ci
Ci
Ci
Ci
Phos,
Ci
Phos
20 mM
10 mM

Figure 117

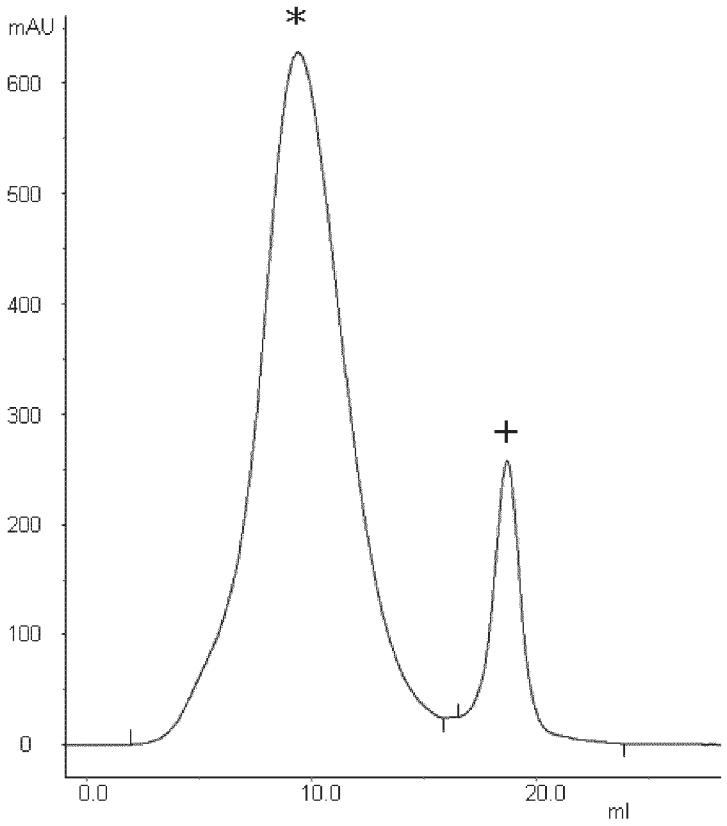


Figure 118

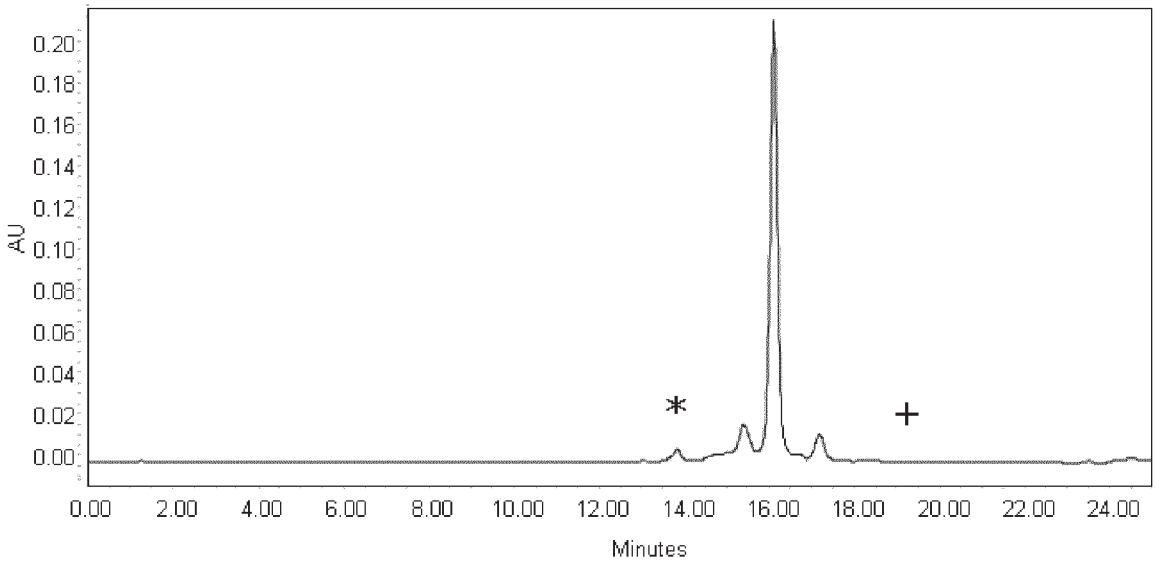


Figure 119

A

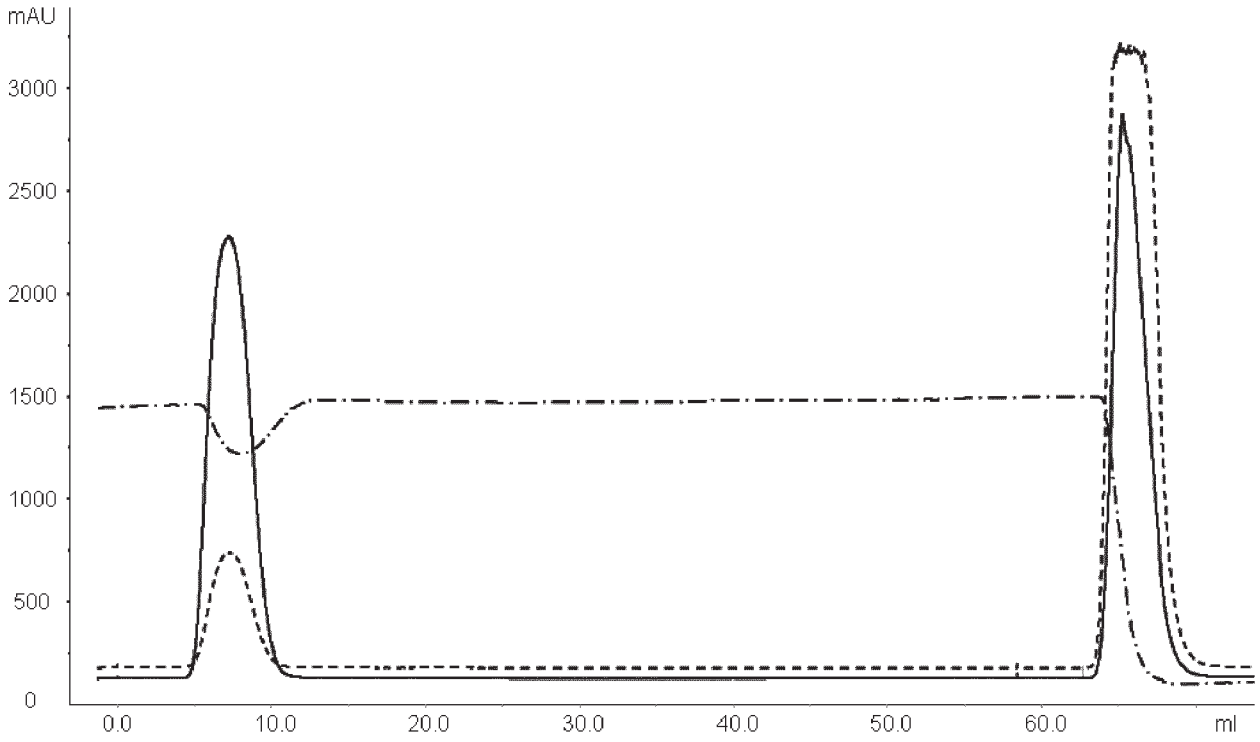


Figure 119

B

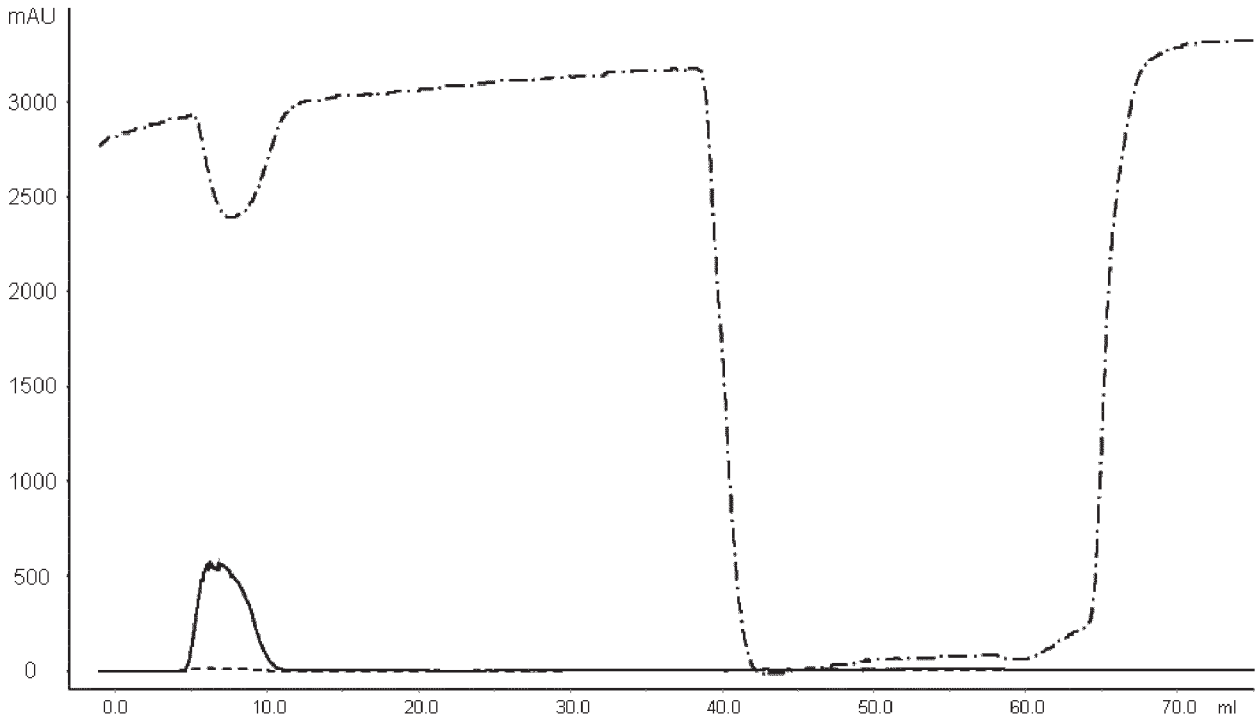


Figure 120 A

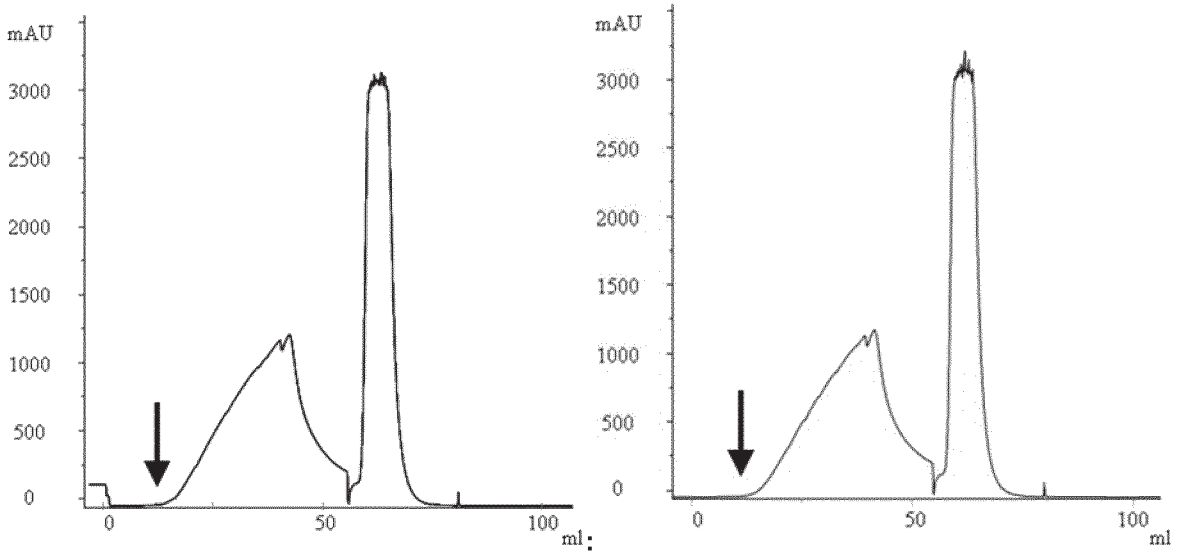


Figure 120 B

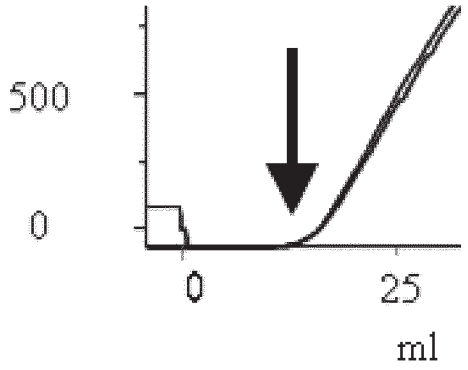


Figure 121

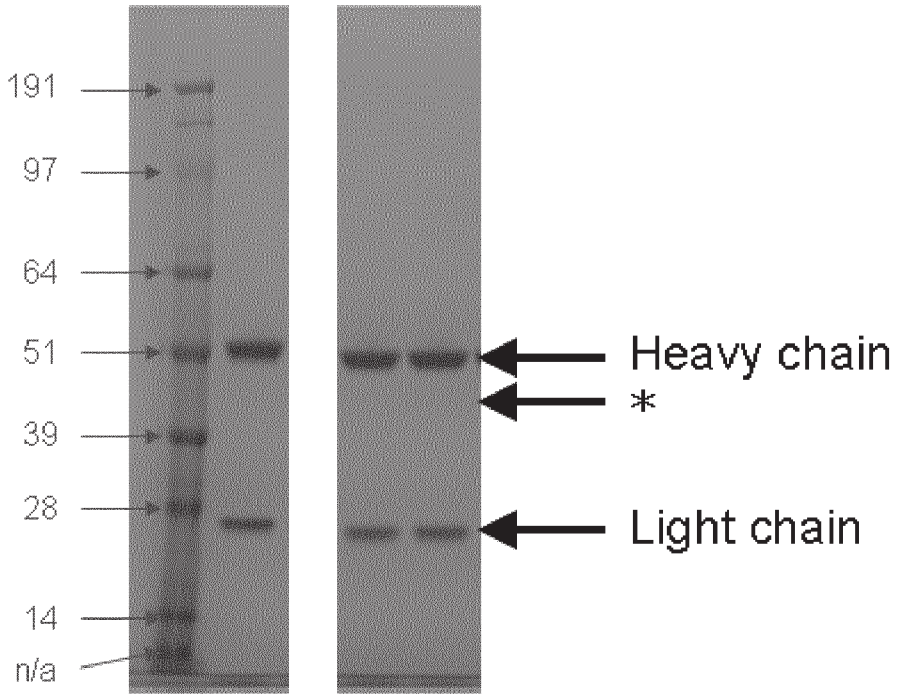


Figure 122

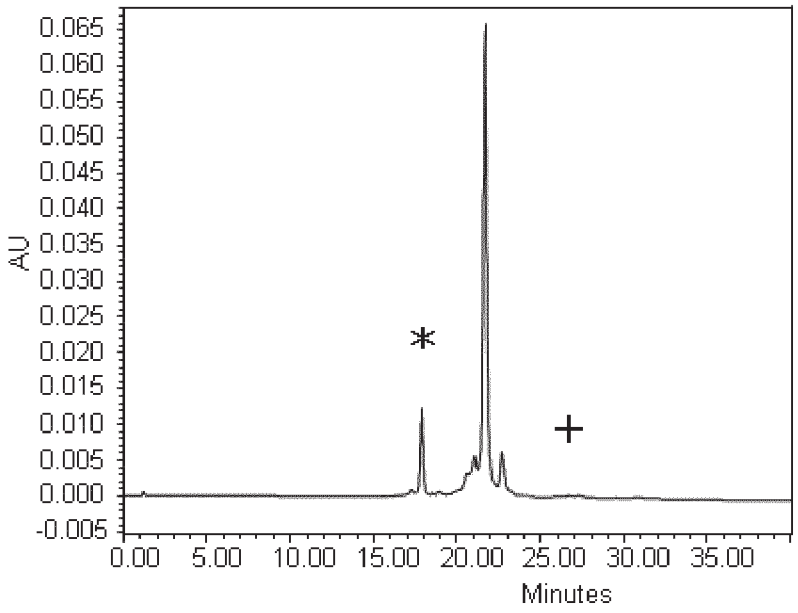
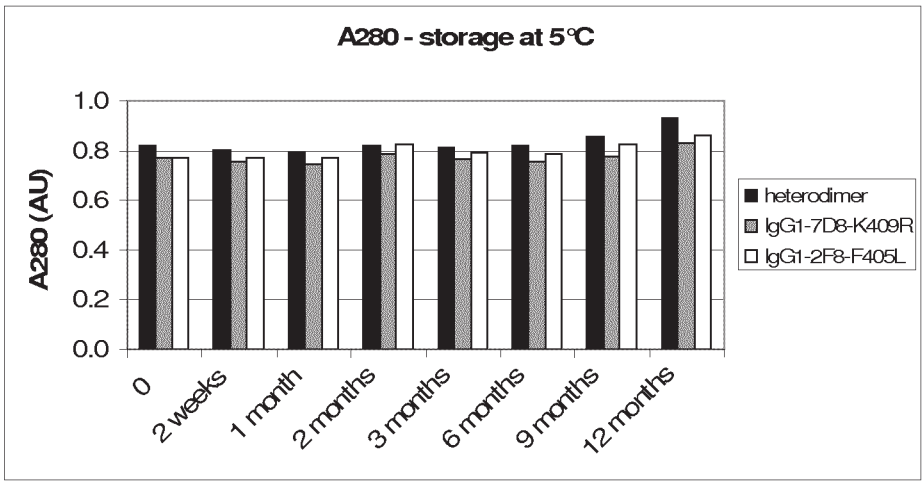
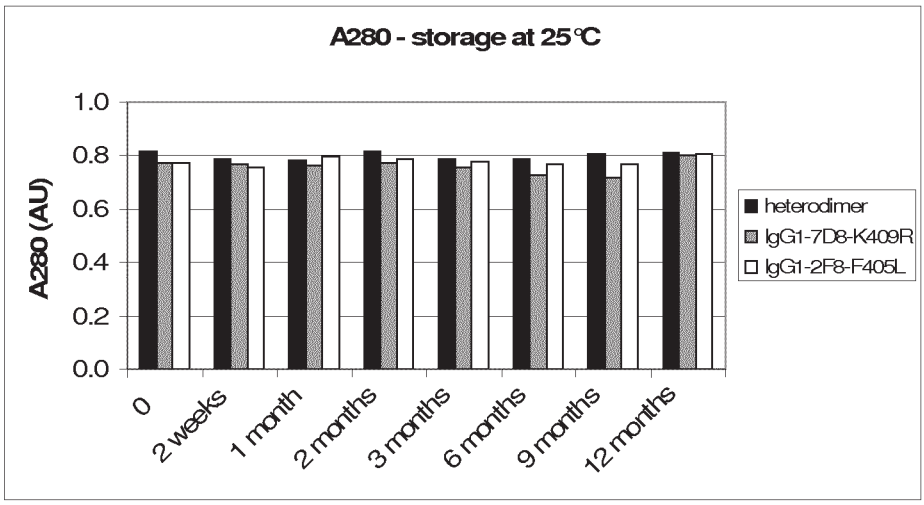

0.065
0.060
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
AU
*
+
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Minutes

Figure 123


A280 - storage at 5°C
A280 (AU)
1.0
0.8
0.6
0.4
0.2
0.0
0
2 weeks
1 month
2 months
3 months
6 months
9 months
12 months
heterodimer
IgG1-7D8-K409R
IgG1-2F8-F405L


A280 - storage at 25°C
A280 (AU)
1.0
0.8
0.6
0.4
0.2
0.0
0
2 weeks
1 month
2 months
3 months
6 months
9 months
12 months
heterodimer
IgG1-7D8-K409R
IgG1-2F8-F405L

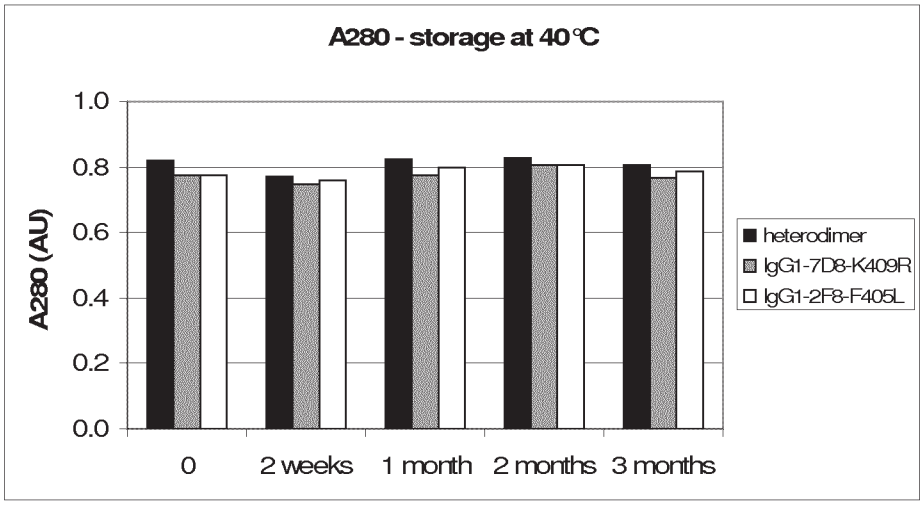

A280 - storage at 40°C
A280 (AU)
1.0
0.8
0.6
0.4
0.2
0.0
0
2 weeks
1 month
2 months
3 months
heterodimer
IgG1-7D8-K409R
IgG1-2F8-F405L

Figure 124

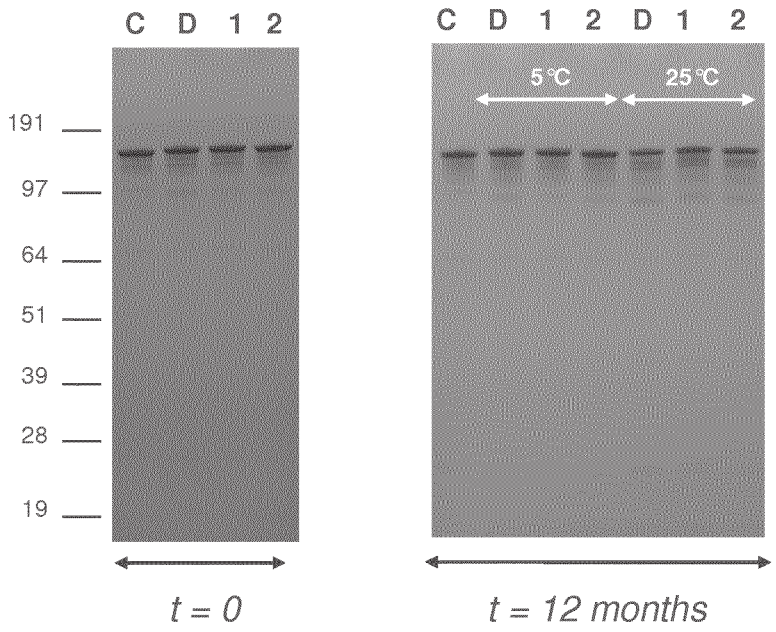


Figure 125

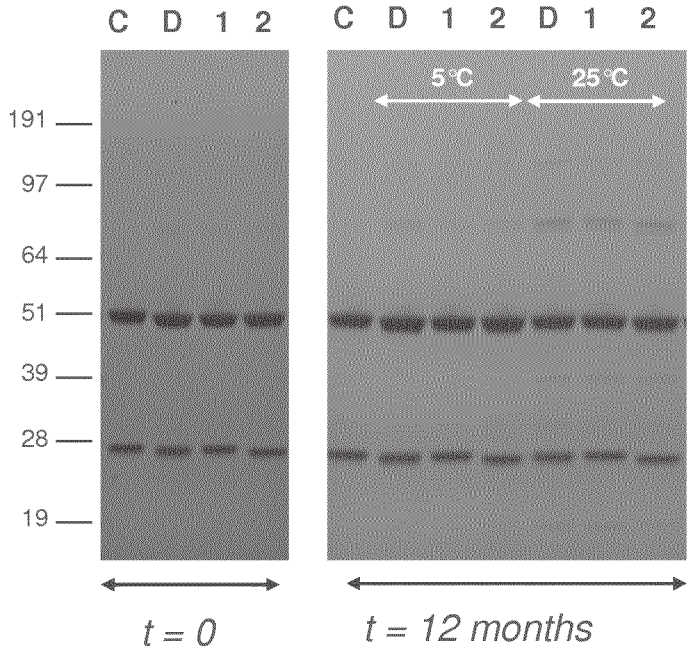


Figure 126

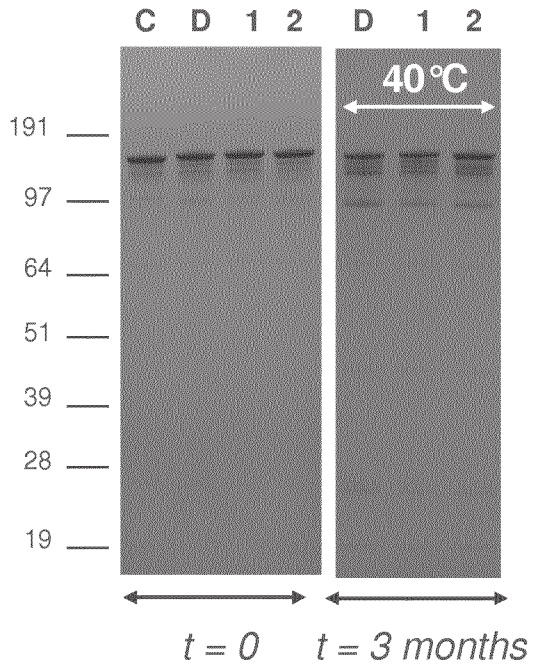


Figure 127

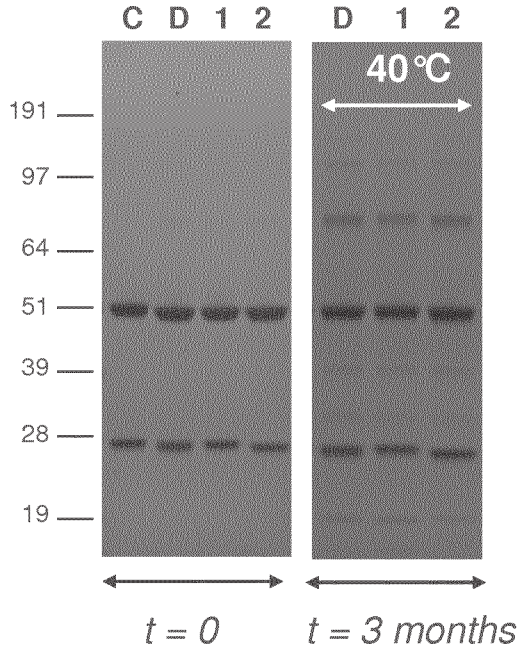


Figure 128

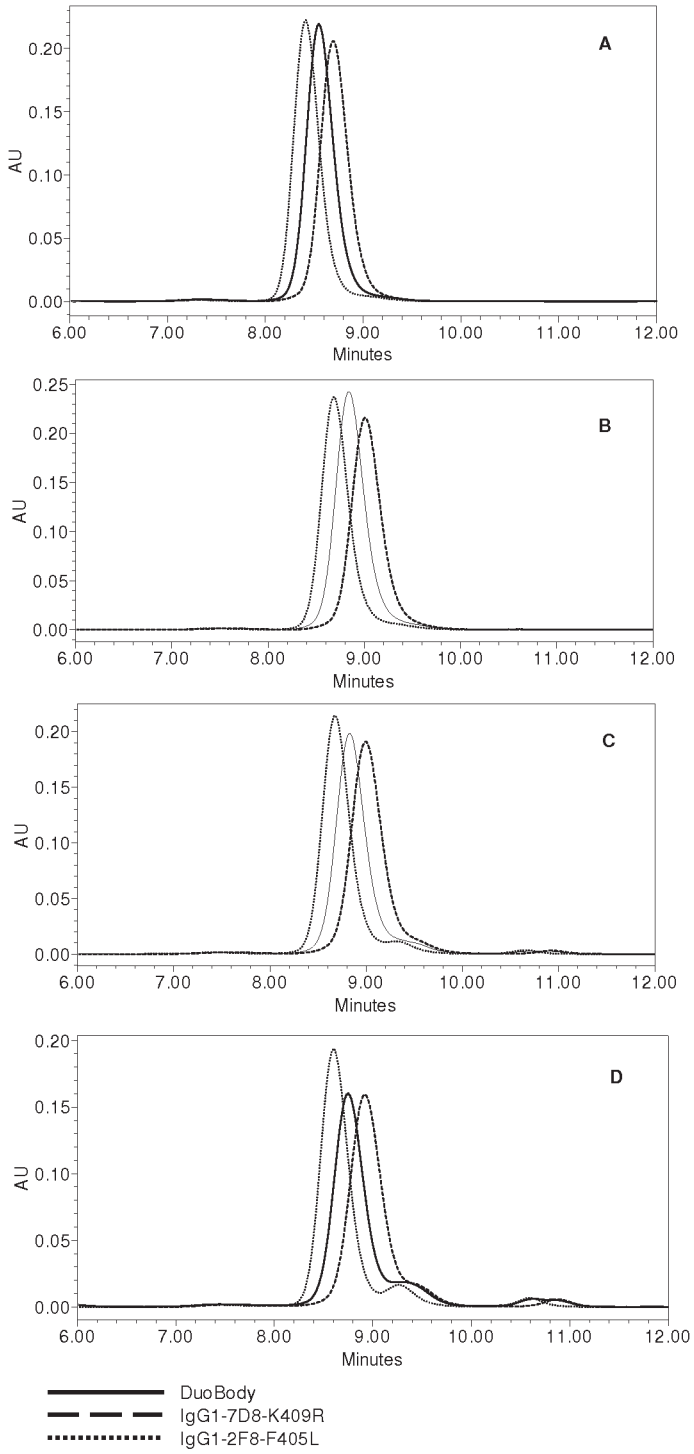
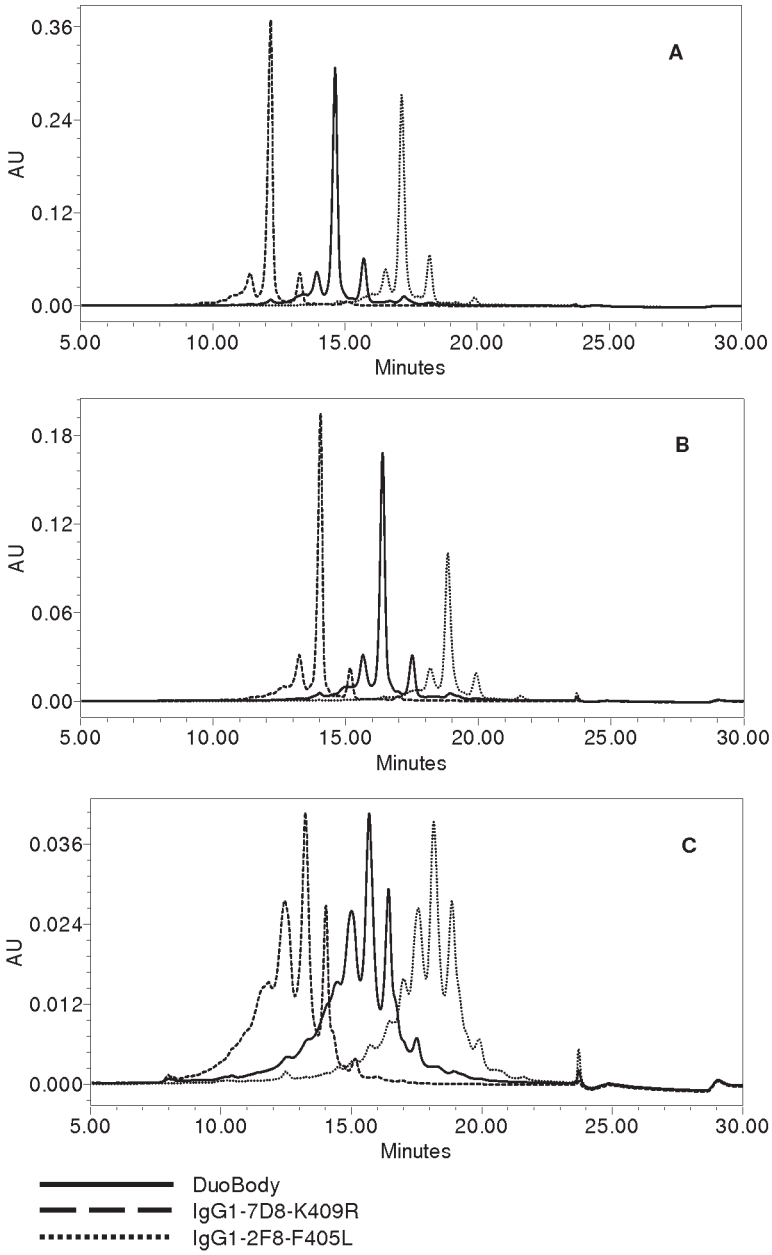

A
B
C
D
AU
Minutes
0.00
0.05
0.10
0.15
0.20
0.25
6.00
7.00
8.00
9.00
10.00
11.00
12.00
DuoBody
IgG1-7D8-K409R
IgG1-2F8-F405L

Figure 129


A
B
C
AU
Minutes
0.36
0.24
0.12
0.00
0.18
0.06
0.036
0.024
0.012
0.000
5.00
10.00
15.00
20.00
25.00
30.00
DuoBody
IgG1-7D8-K409R
IgG1-2F8-F405L