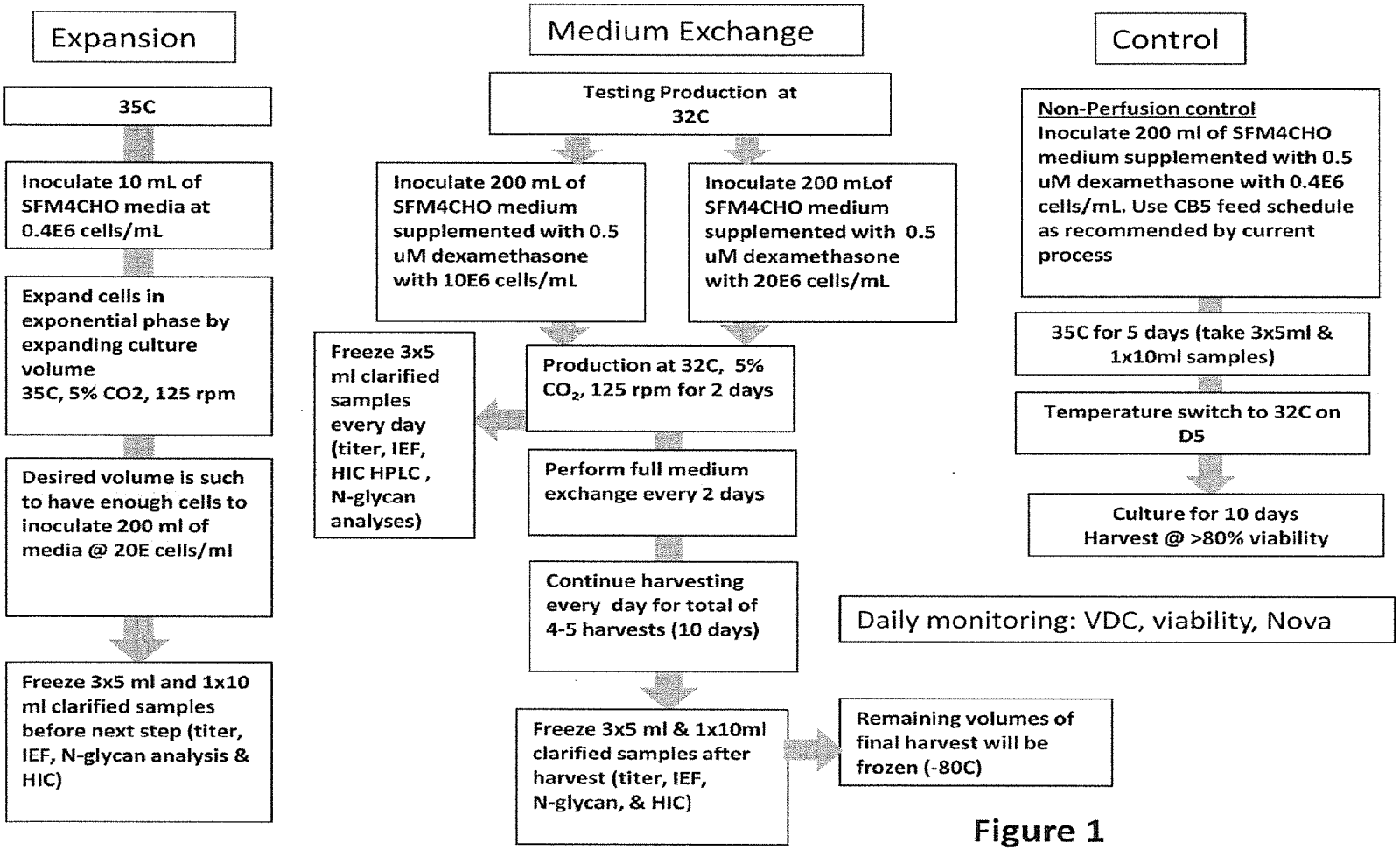
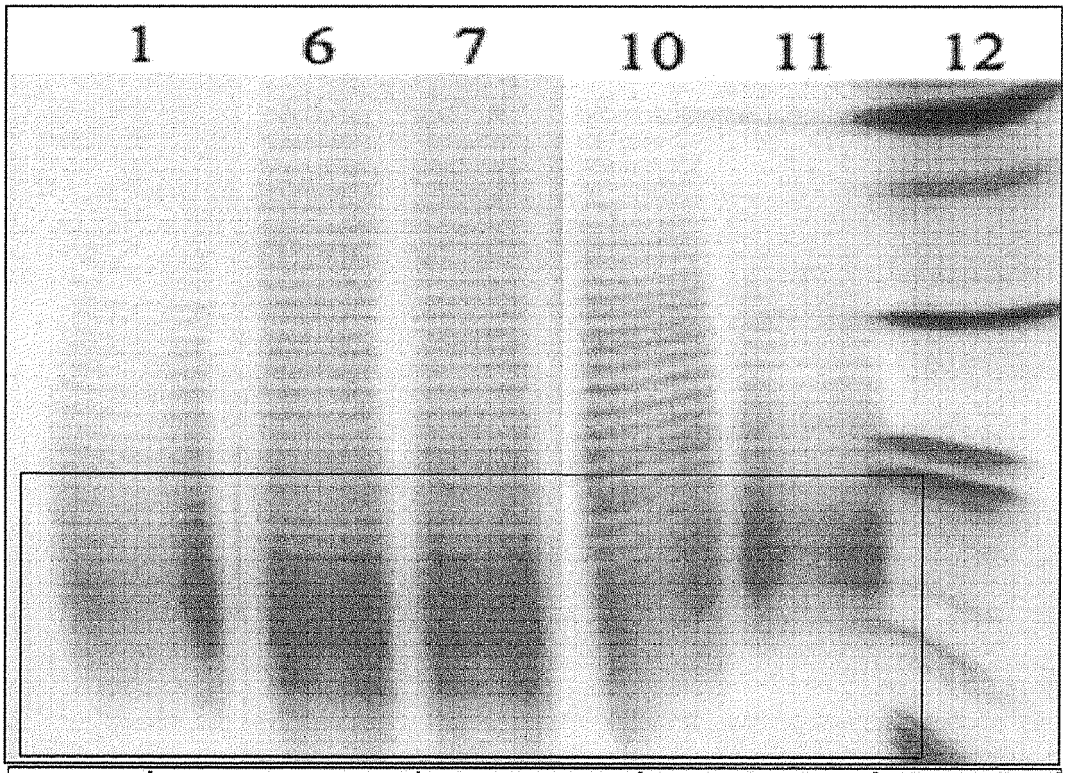

Expansion
35C
Inoculate 10 mL of SFM4CHO media at 0.4E6 cells/mL
Expand cells in exponential phase by expanding culture volume
35C, 5% CO2, 125 rpm
Desired volume is such to have enough cells to inoculate 200 ml of media @ 20E cells/ml
Freeze 3x5 ml and 1x10 ml clarified samples before next step (titer, IEF, N-glycan analysis & HIC)
Medium Exchange
Testing Production at 32C
Inoculate 200 mL of SFM4CHO medium supplemented with 0.5 uM dexamethasone with 10E6 cells/mL
Inoculate 200 mLof SFM4CHO medium supplemented with 0.5 uM dexamethasone with 20E6 cells/mL
Freeze 3x5 ml clarified samples every day (titer, IEF, HIC HPLC , N-glycan analyses)
Production at 32C, 5% CO_2, 125 rpm for 2 days
Perform full medium exchange every 2 days
Continue harvesting every day for total of 4-5 harvests (10 days)
Freeze 3x5 ml & 1x10ml clarified samples after harvest (titer, IEF, N-glycan, & HIC)
Remaining volumes of final harvest will be frozen (-80C)
Control
Non-Perfusion control
Inoculate 200 ml of SFM4CHO medium supplemented with 0.5 uM dexamethasone with 0.4E6 cells/mL. Use CB5 feed schedule as recommended by current process
35C for 5 days (take 3x5ml & 1x10ml samples)
Temperature switch to 32C on D5
Culture for 10 days
Harvest @ >80% viability
Daily monitoring: VDC, viability, Nova

Figure 1



Gel sample	Sample	Titer (mg/L)	maxVCD (cE6/ml)	Viability (%)
1	Enbrel ctl			
6	20E6c/ml; Harvest #2	163	15.6	98
7	10E6c/ml; Harvest #2	298	10.2	98
10	10E6c/ml; Harvest #3	320	9.4	97
11	Enbrel ctl			
12	pI Marker			

FIGURE 2

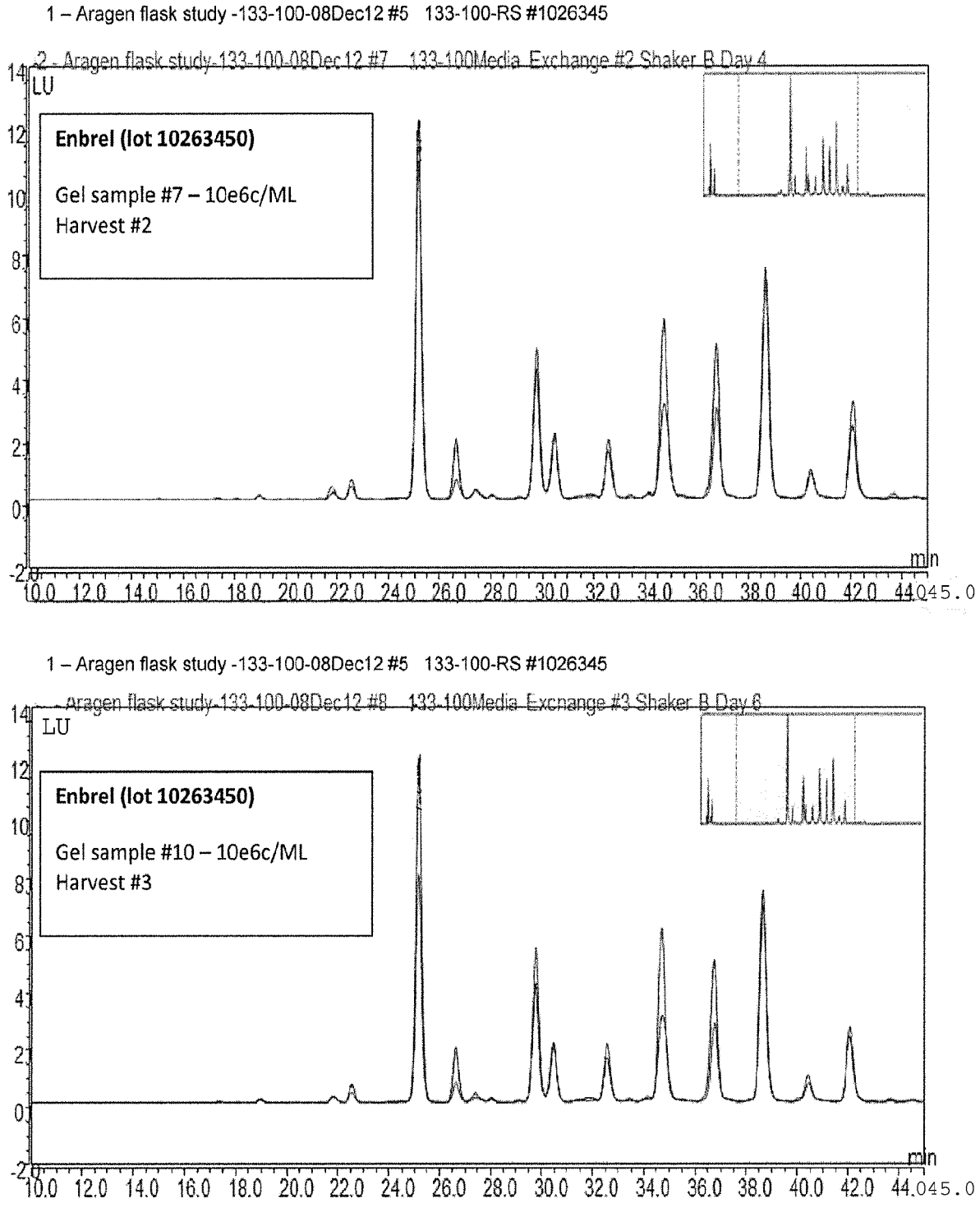

1 – Aragen flask study -133-100-08Dec12 #5 133-100-RS #1026345
2 - Aragen flask study-133-100-08Dec12 #7 133-100Media Exchange #2 Shaker B Day 4
LU
Enbrel (lot 10263450)
Gel sample #7 – 10e6c/ML
Harvest #2
min
1 – Aragen flask study -133-100-08Dec12 #5 133-100-RS #1026345
Aragen flask study-133-100-08Dec12 #8 133-100Media Excnange #3 Shaker B Day 6
LU
Enbrel (lot 10263450)
Gel sample #10 – 10e6c/ML
Harvest #3
min

FIGURE 3

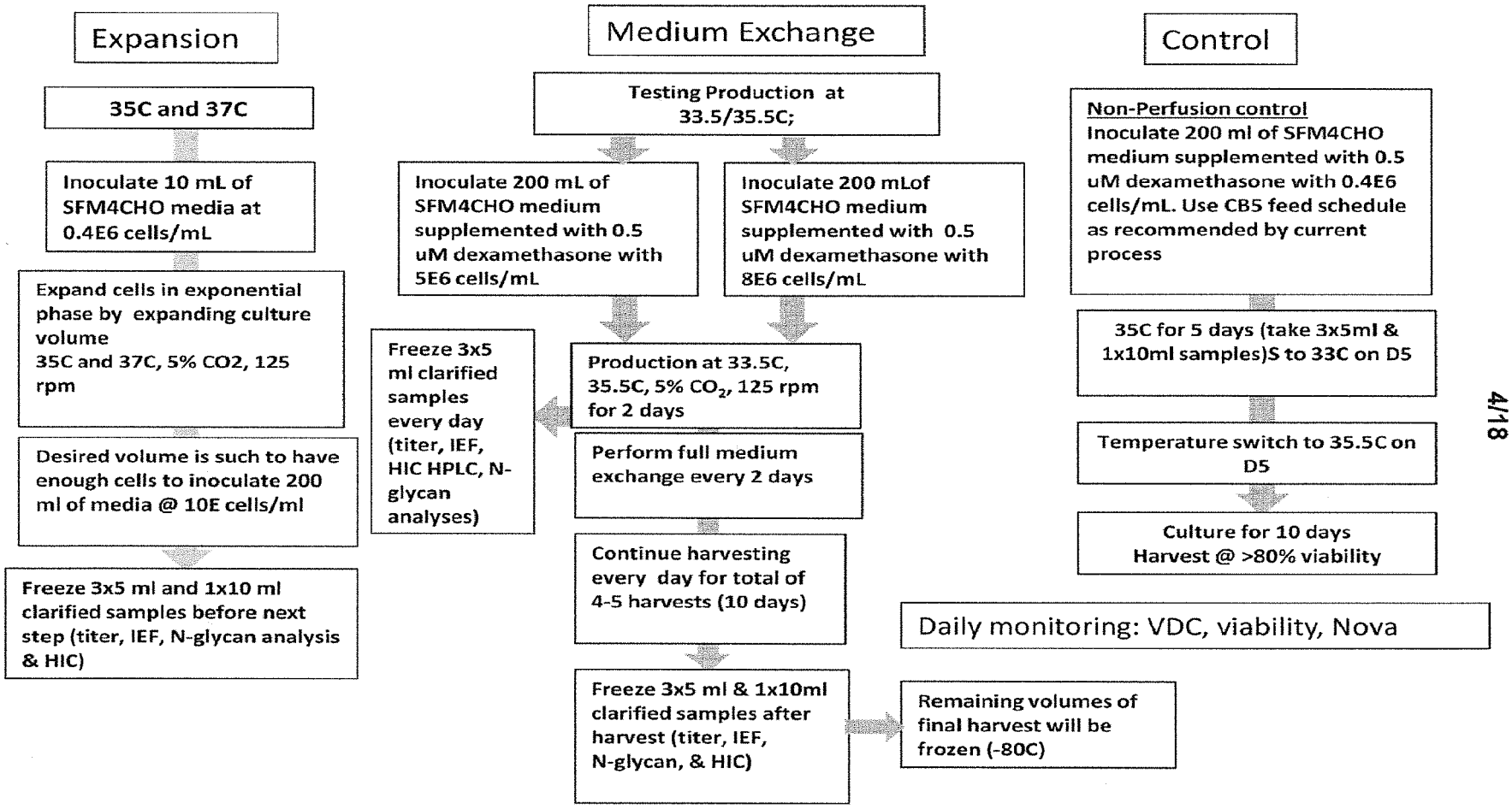
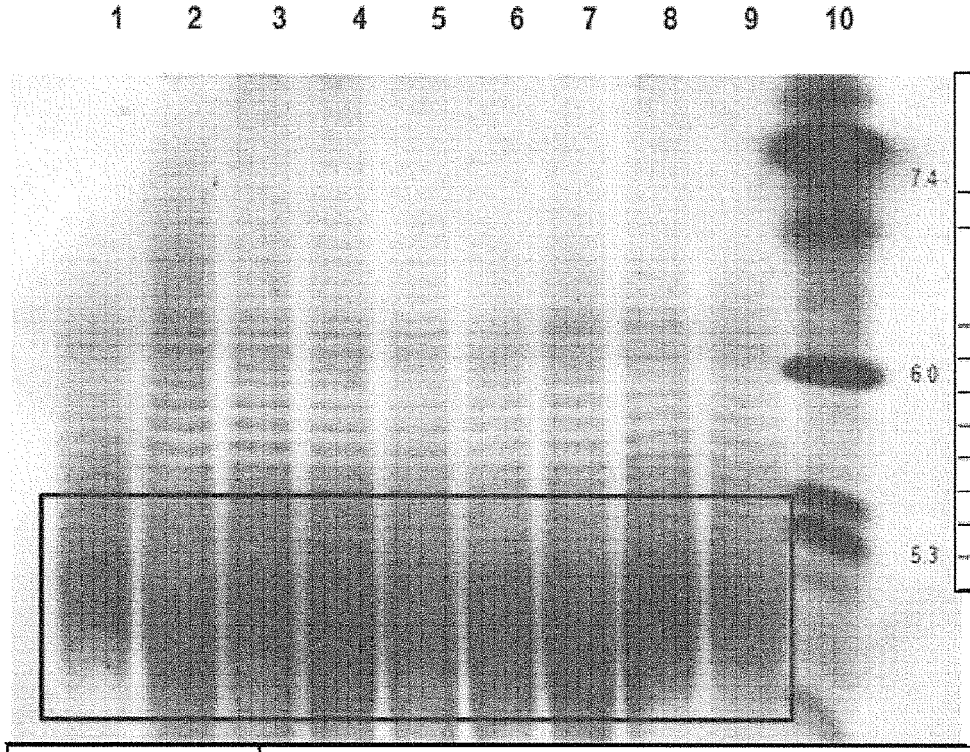

Expansion
35C and 37C
Inoculate 10 mL of SFM4CHO media at 0.4E6 cells/mL
Expand cells in exponential phase by expanding culture volume
35C and 37C, 5% CO2, 125 rpm
Desired volume is such to have enough cells to inoculate 200 ml of media @ 10E cells/ml
Freeze 3x5 ml and 1x10 ml clarified samples before next step (titer, IEF, N-glycan analysis & HIC)
Medium Exchange
Testing Production at 33.5/35.5C;
Inoculate 200 mL of SFM4CHO medium supplemented with 0.5 uM dexamethasone with 5E6 cells/mL
Inoculate 200 mLof SFM4CHO medium supplemented with 0.5 uM dexamethasone with 8E6 cells/mL
Freeze 3x5 ml clarified samples every day (titer, IEF, HIC HPLC, N-glycan analyses)
Production at 33.5C, 35.5C, 5% CO_2, 125 rpm for 2 days
Perform full medium exchange every 2 days
Continue harvesting every day for total of 4-5 harvests (10 days)
Freeze 3x5 ml & 1x10ml clarified samples after harvest (titer, IEF, N-glycan, & HIC)
Remaining volumes of final harvest will be frozen (-80C)
Control
Non-Perfusion control
Inoculate 200 ml of SFM4CHO medium supplemented with 0.5 uM dexamethasone with 0.4E6 cells/mL. Use CB5 feed schedule as recommended by current process
35C for 5 days (take 3x5ml & 1x10ml samples)S to 33C on D5
Temperature switch to 35.5C on D5
Culture for 10 days
Harvest @ >80% viability
Daily monitoring: VDC, viability, Nova

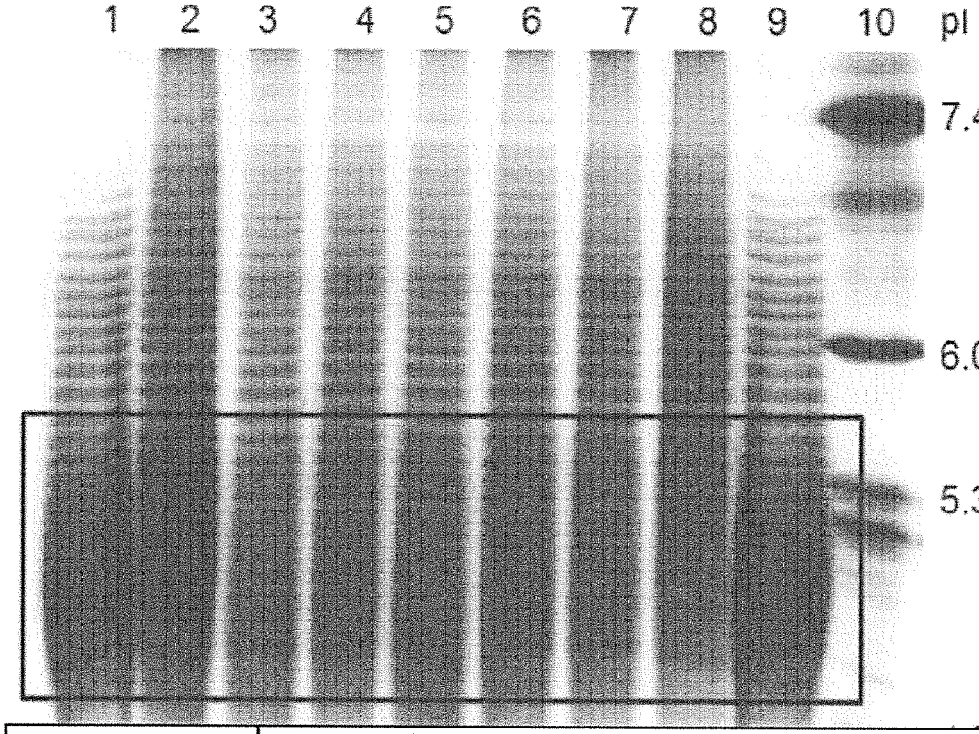
Figure 4



Lane #	Sample	Expansion Temp.(C)	Production Temp.(C)	Intended Inoculum (106 cells/ml)
1	Enbrel			
2	Control (day 2 after T switch)	35	35.5	0.4
3	Shaker #1	37	35.5	5
4	Shaker #2	37	35.5	8
5	Shaker #3	35	33.5	5
6	Shaker #4	35	33.5	8
7	Shaker #5	35	35.5	5
8	Shaker #6	35	35.5	9
9	Enbrel			
10	pI Marker			

	Sample						
	2	3	4	5	6	7	8
Titer (ug/ml)	371	209	234	172	155	177	198
VCD (10E6 cells/ml)	6.9	6.6	8.7	7.2	8.7	7.8	10.2
Viability (%)	99	98	98	99	99	99	99

FIGURE 5



Lane #	Sample	Expansion Temp.(C)	Production Temp.(C)	Intended Inoculum (106 cells/ml)
1	Enbrel			
2	Control (day 6 after T switch)	35	35.5	0.4
3	Shaker #1	37	35.5	5
4	Shaker #2	37	35.5	8
5	Shaker #3	35	33.5	5
6	Shaker #4	35	33.5	8
7	Shaker #5	35	35.5	5
8	Shaker #6	35	35.5	9
9	Enbrel			
10	pI Marker			

	Sample						
	2	3	4	5	6	7	8
Titer (ug/ml)	930	551	570	478	637	338	676
VCD (10E6 cells/ml)	6.5	9.5	10	9	10.2	8.5	8.6
Viability (%)	93.7	94.3	93	92.4	92.5	87.5	83.8

FIGURE 6

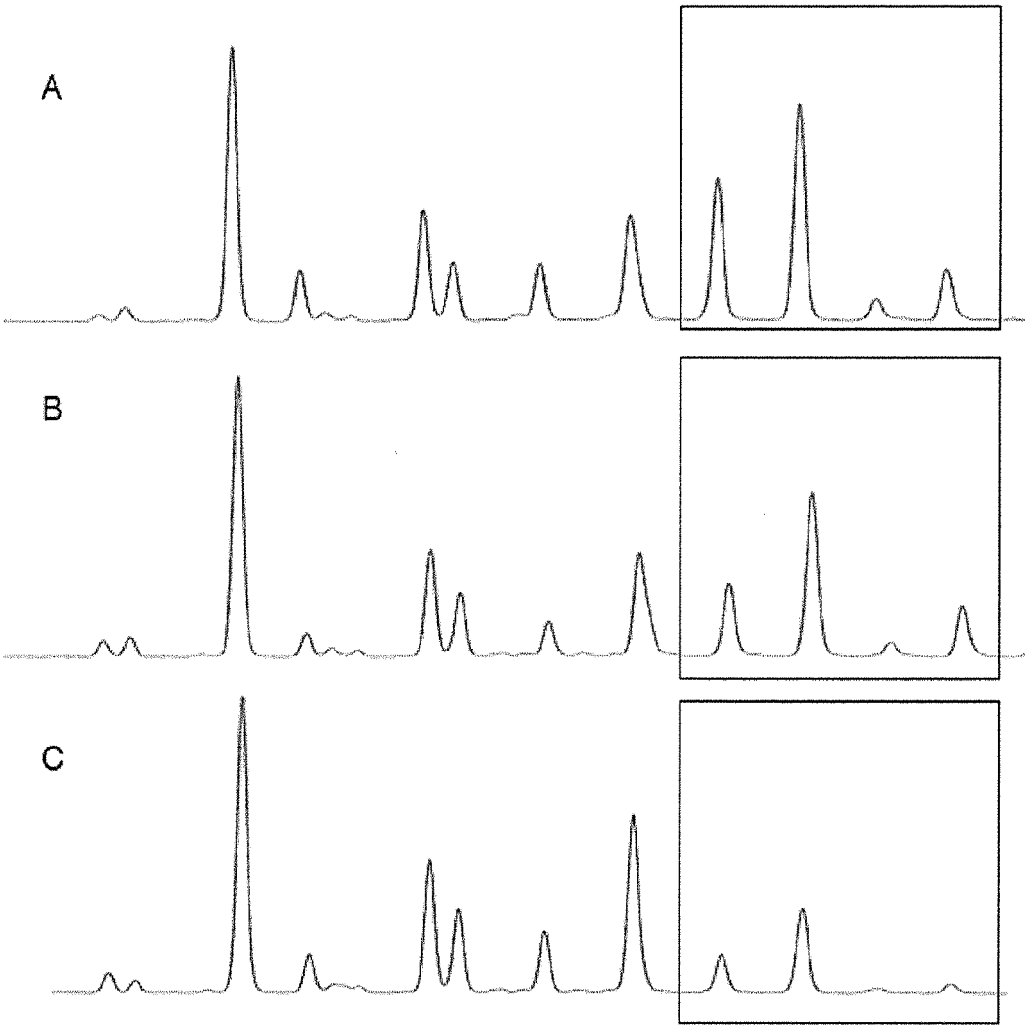
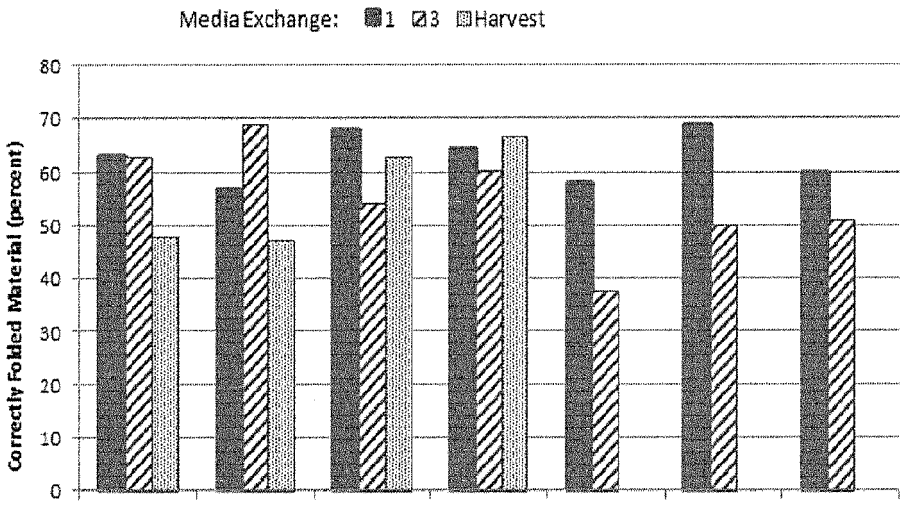


FIGURE 7

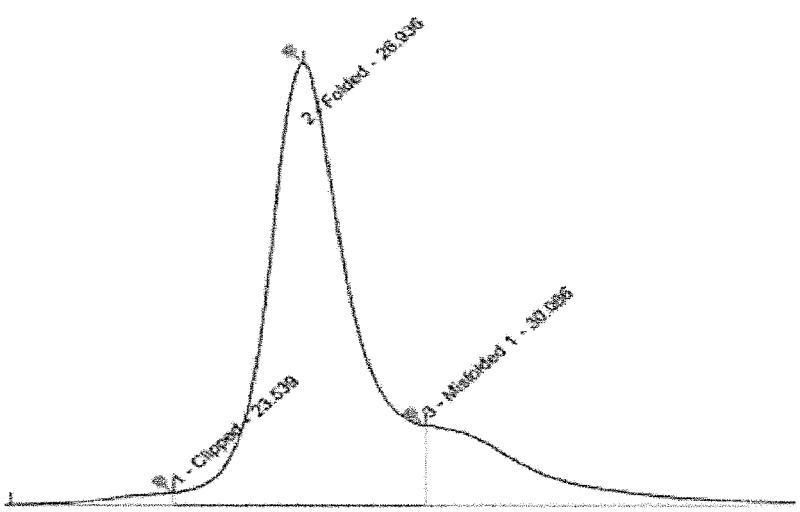


Growth Temp (°C)	37	37	35	35	35	35	35
Production Temp (°C)	33.5	33.5	33.5	33.5	35.5	35.5	35.5
Seeding Density (x 10^6)	5	8	5	8	5	8	0.4
	SF1	SF2	SF3	SF4	SF5	SF6	Control

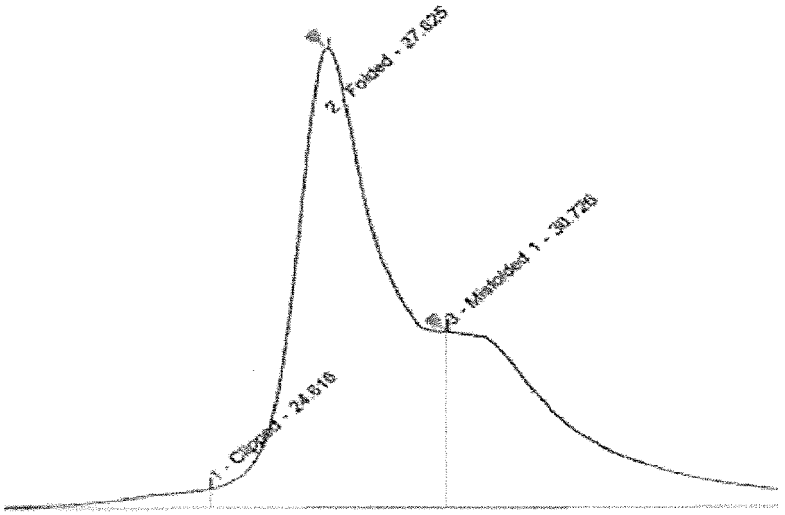
Cultures were expanded under the conditions indicated (growth temperature), and product was produced (production temperature) in shake flasks seeded at the indicated concentrations. Bars indicated percent of material in the peak representing correctly folded product from chromatograms such as those shown in Figure 9 through 13.

FIGURE 8

A



B



FIGURES 9 through 13 (chromatograms A through j) depict the results of Hydrophobic Interaction Chromatography (HIC) of selected samples from media exchange study with respect to etanercept produced according to examples 1 and 2. Samples were subjected to HIC analysis after media exchange or harvest. Clipped, correctly folded, and misfolded product is indicated for each chromatogram. Integrations of the correctly folded peak are represented by the bar chart in Figure 8.

FIGURE 9

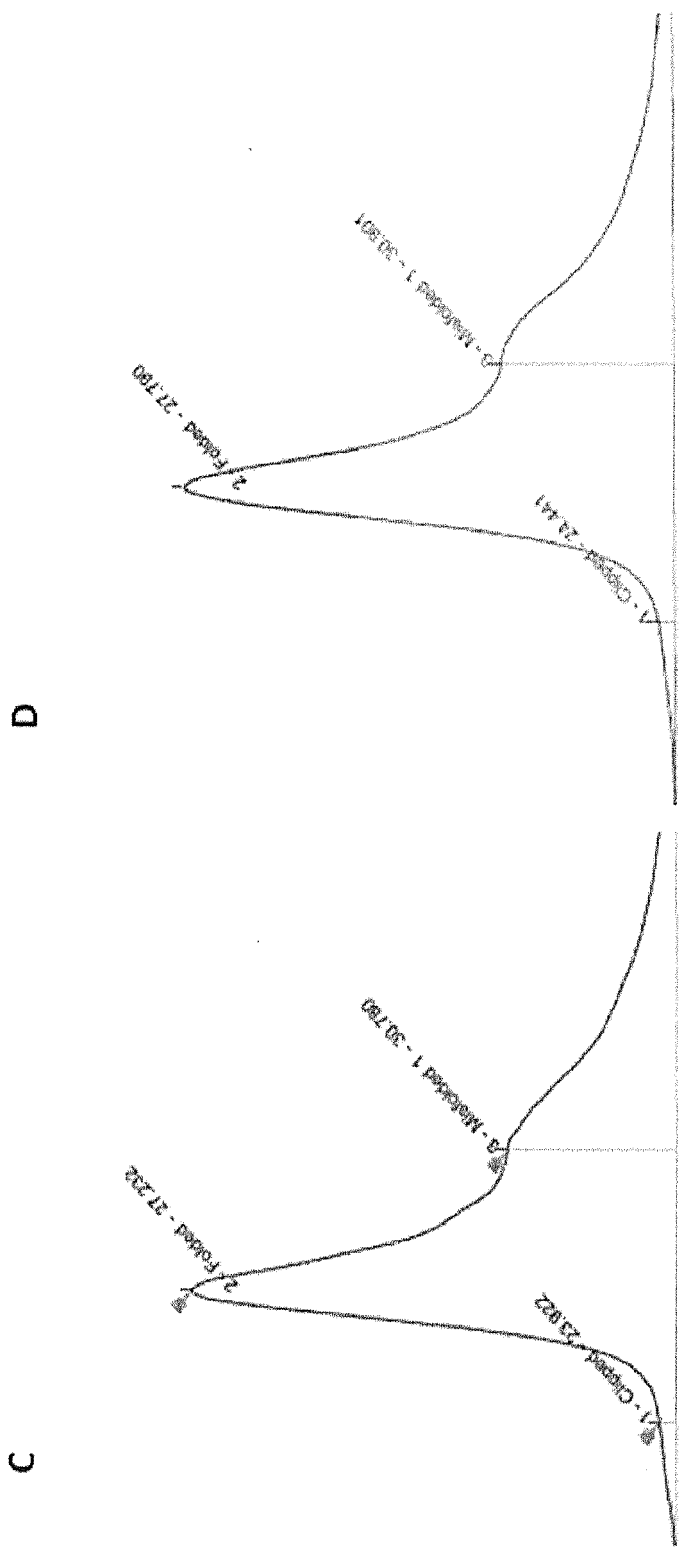

C
1 - Clopidol - 23.022
2 - Fobidol - 27.202
3 - Halofuginol 1 - 30.780
D
1 - Clopidol - 23.441
2 - Fobidol - 27.780
3 - Halofuginol 1 - 30.981

FIGURE 10

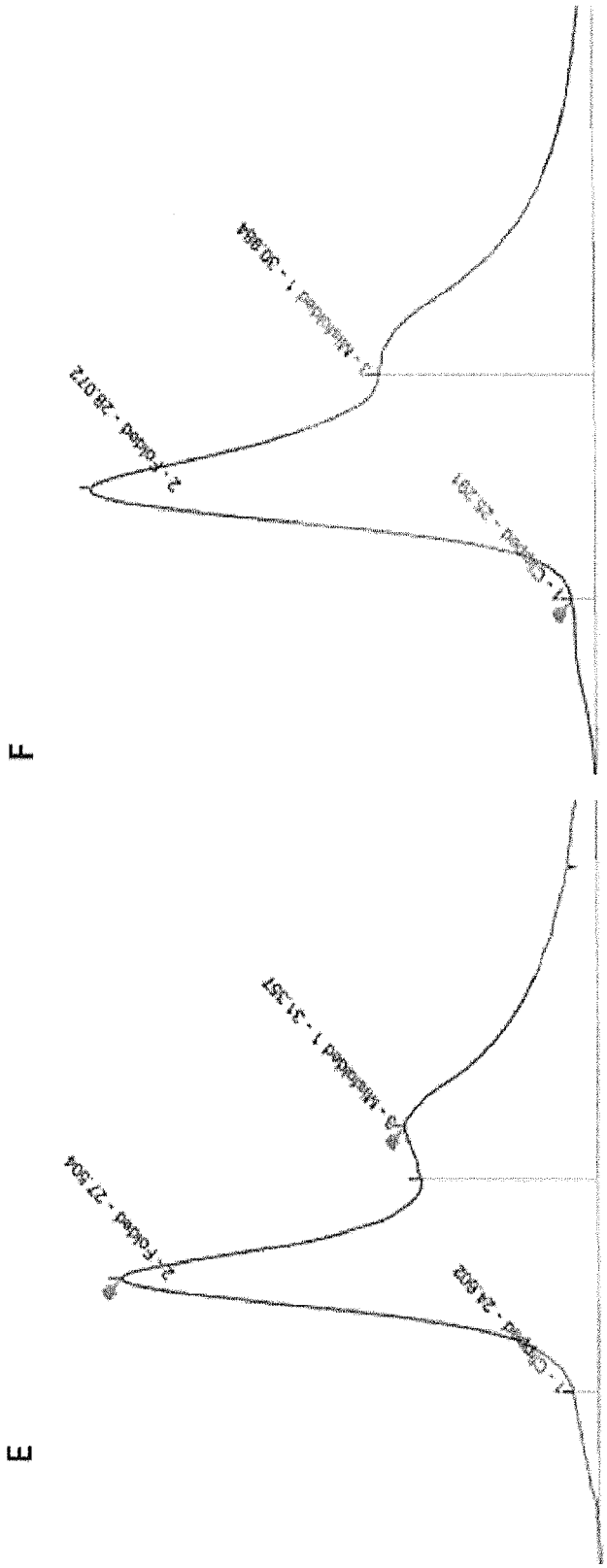

F
1 - Clipped - 28.291
2 - Folded - 28.012
3 - Unfolded 1 - 30.884
E
1 - Clipped - 24.672
2 - Folded - 27.504
3 - Unfolded 1 - 31.357

FIGURE 11

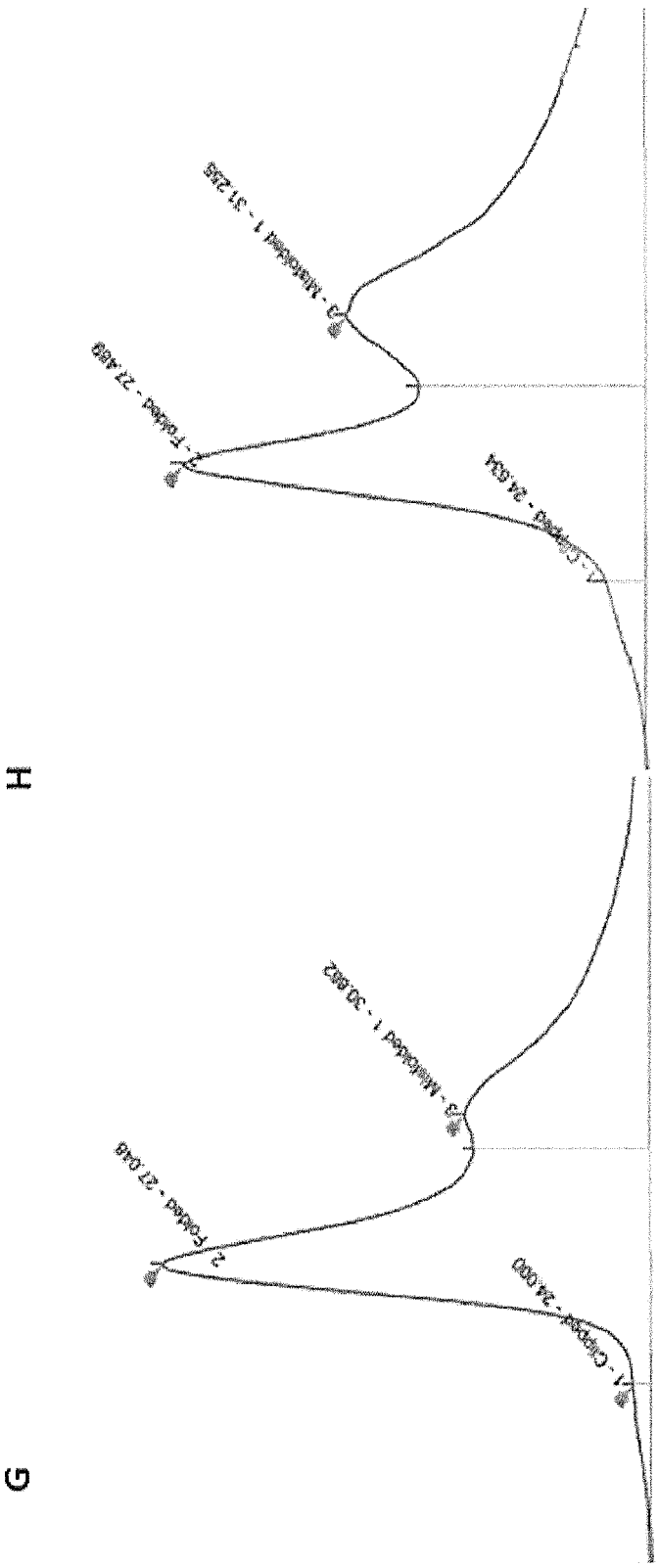

H
1 - Clopidol - 24.654
2 - Fluidol - 27.489
3 - Maduramicin 1 - 31.235
G
1 - Clopidol - 24.000
2 - Fluidol - 27.049
3 - Maduramicin 1 - 30.962

FIGURE 12

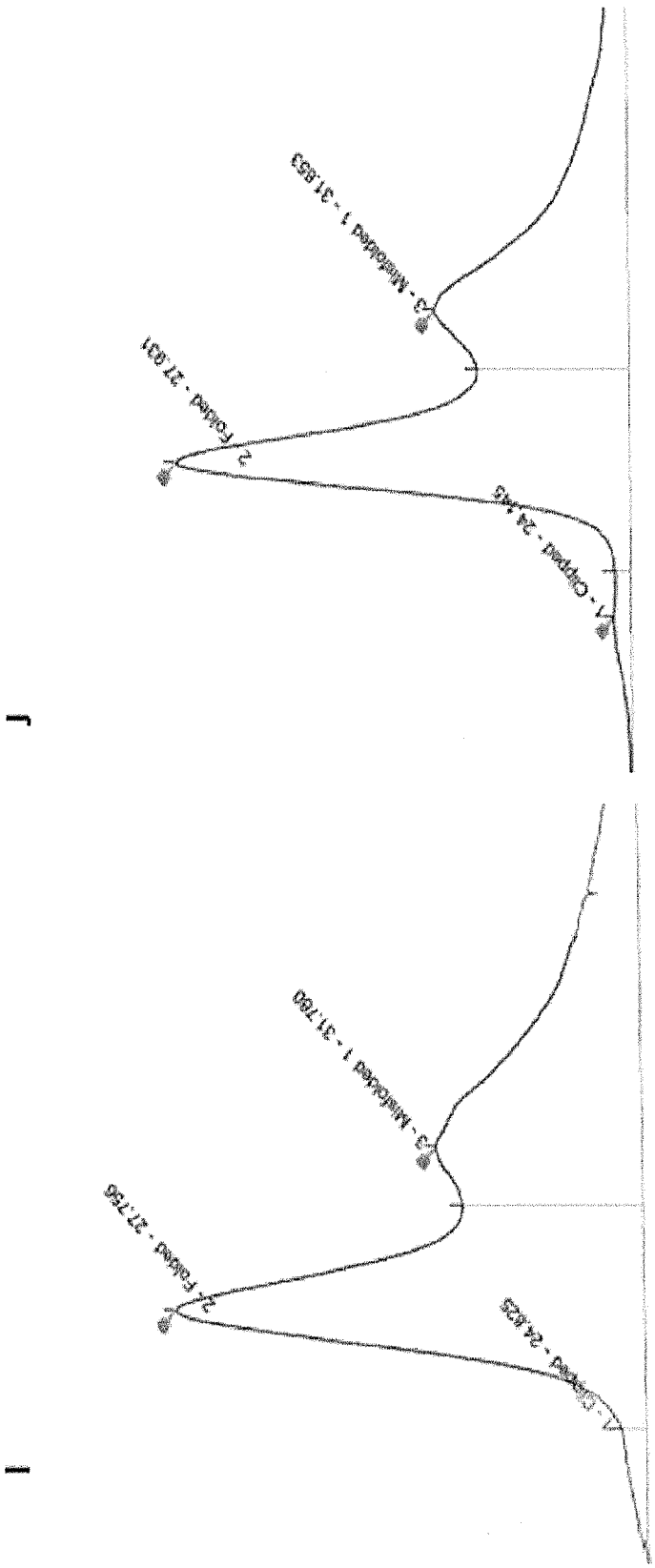

I
1 - Diclofenac - 24.825
2 - Folded - 27.750
3 - Misfolded 1 - 31.750
J
1 - Cytopur - 24.463
2 - Folded - 27.021
3 - Misfolded 1 - 31.853

FIGURE 13

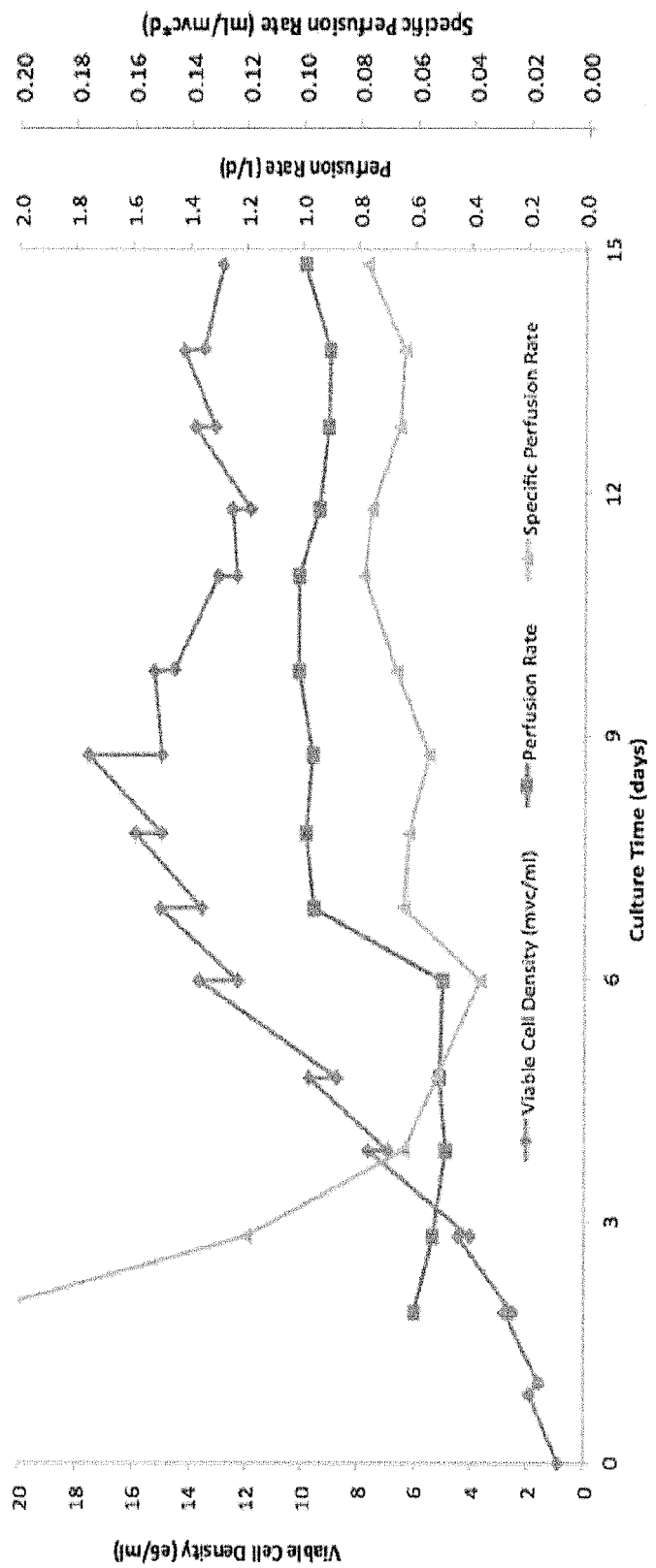


FIGURE 14

FIGURE 14

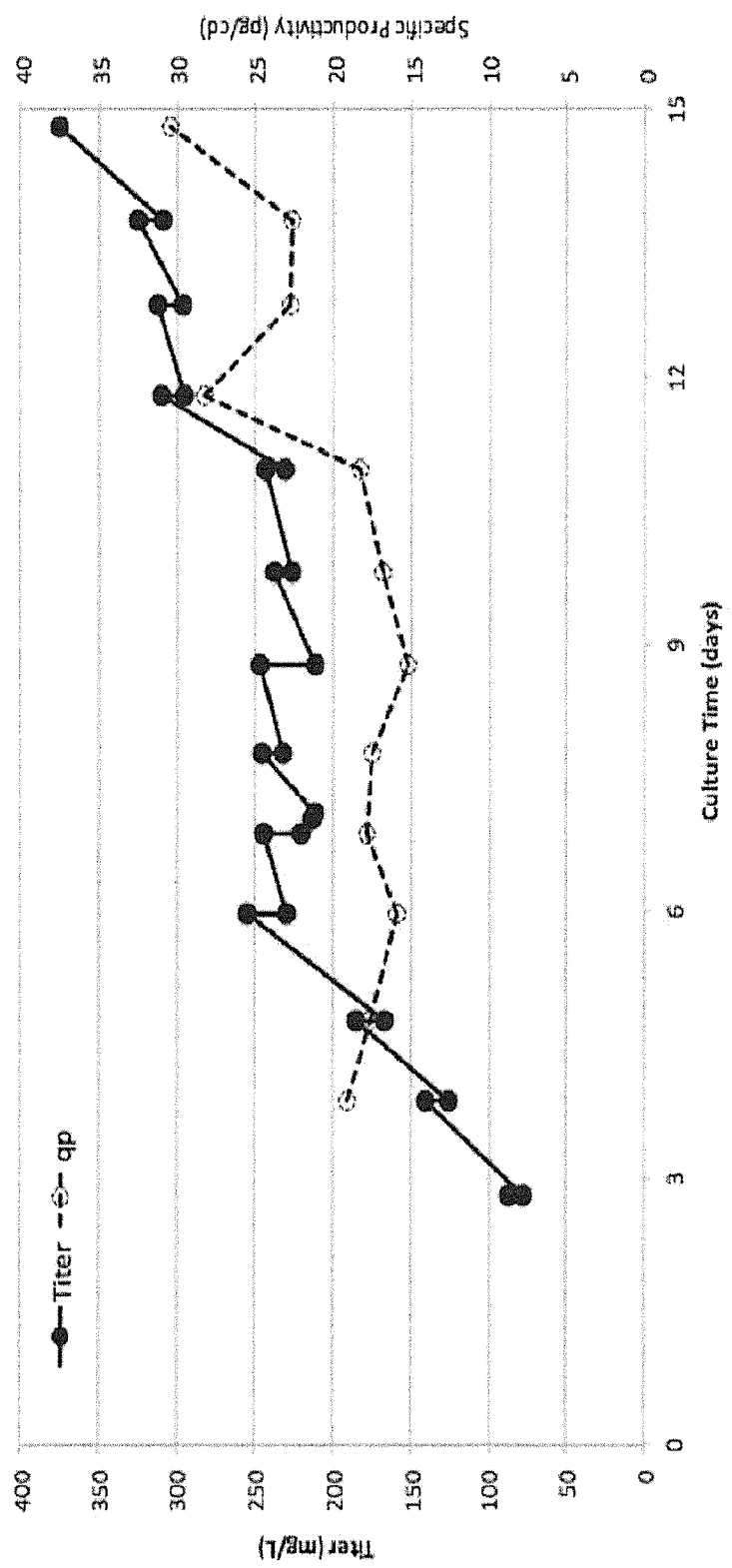
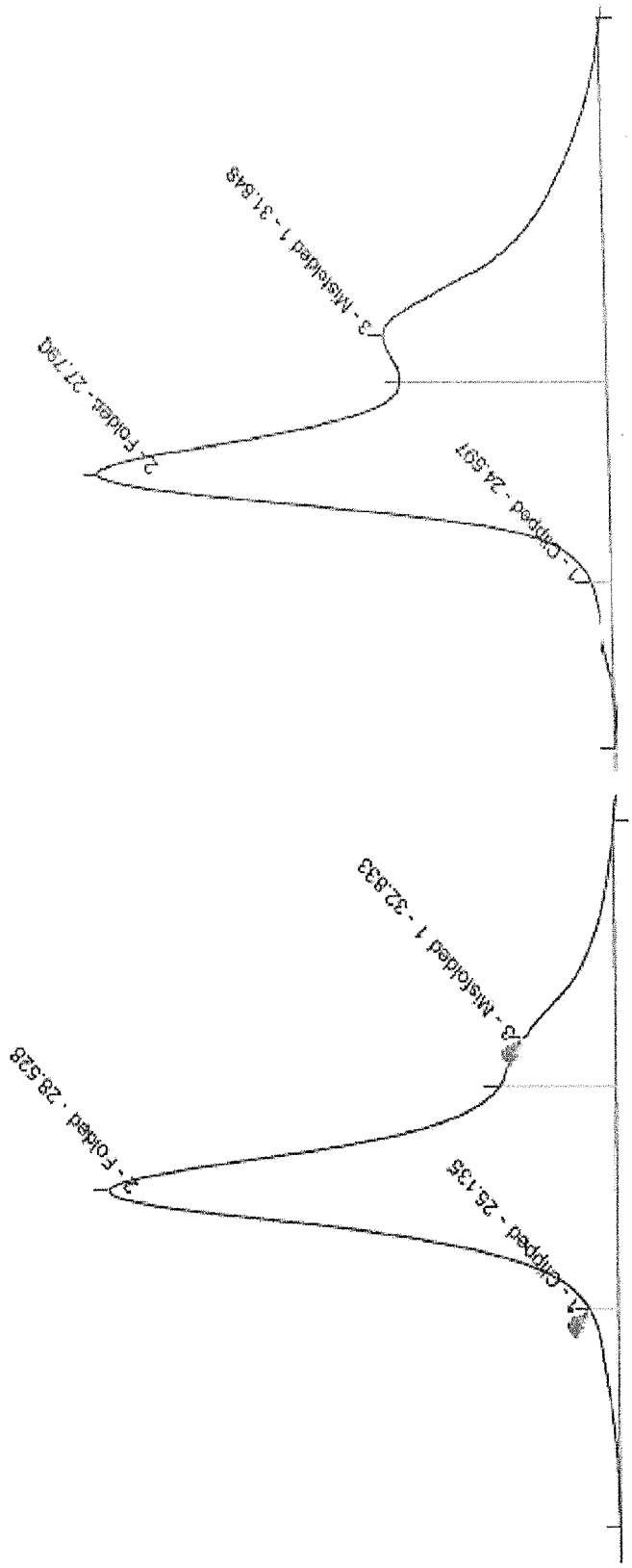

Titer (mg/L)
Specific Productivity (pg/cd)
Culture Time (days)
Titer
qp
0
50
100
150
200
250
300
350
400
0
5
10
15
20
25
30
35
40
0
3
6
9
12
15

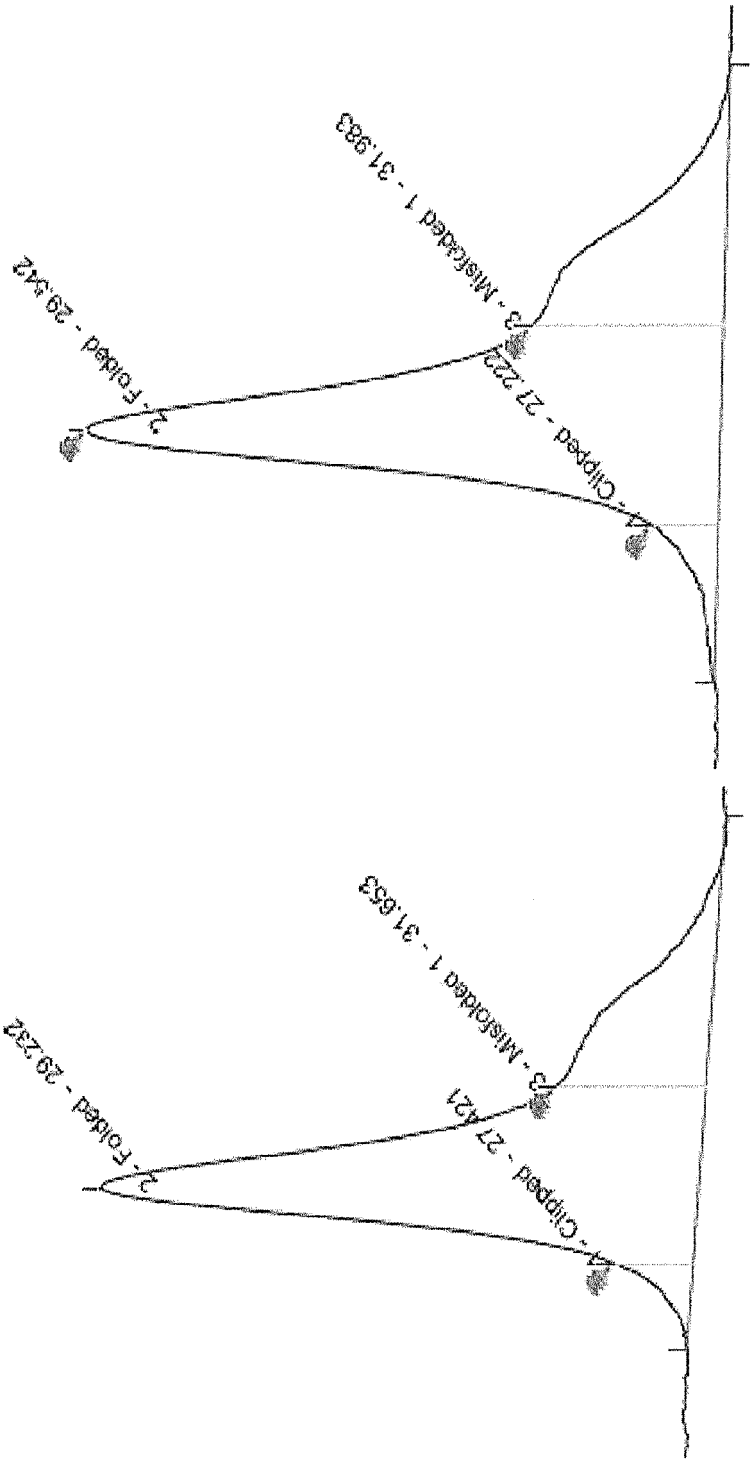
FIGURE 15


1 - Clipped - 25.135
2 - Folded - 28.528
3 - Misfolded 1 - 32.833
1 - Clipped - 24.697
2 - Folded - 27.790
3 - Misfolded 1 - 31.849

A B

FIGURE 16

FIGURE 16 (continued)



C D

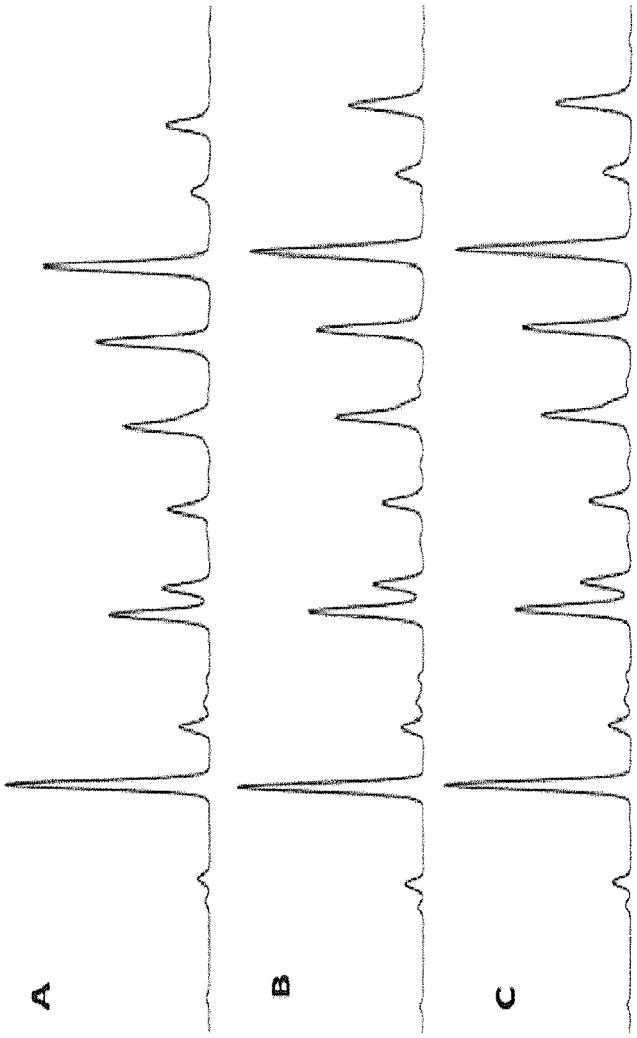


FIGURE 17