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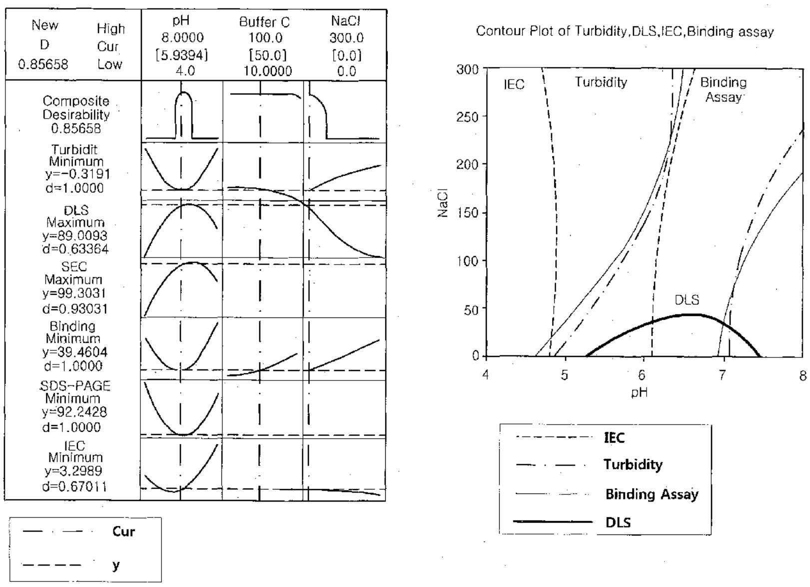
(54) **Title**: PHARMACEUTICAL FORMULATION COMPRISING ANTI-EGFR ANTIBODY

Fig. 19



(57) **Abstract**: The present invention provides a pharmaceutical formulation comprising an anti- epidermal growth factor receptor (EGFR) antibody. The pharmaceutical formulation has low turbidity, without showing aggregation or particle formation, even under accelerated conditions, and exhibits good stability. Therefore, the pharmaceutical formulation can be effectively used for the treatment of disorders such as cancer.

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Description

Title of Invention

PHARMACEUTICAL FORMULATION COMPRISING ANTI-EGFR ANTIBODY

Technical Field

The present invention relates to a pharmaceutical formulation comprising an anti-EGFR antibody.

Background Art

An antibody binding to epidermal growth factor receptor (EGFR) is called an anti-EGFR antibody. Examples of such antibody include cetuximab which is a chimeric antibody containing a variable region of mouse origin and a constant region of human origin (Naramura et al., *Cancer lmmunol. Immunotherapy*, 1993, 37: 343-349, and International Publication NO. WO 96/40210), and MAB 425 which is an original mouse antibody to EGFR (Kettleborough et al., *Protein Engineering*, 1991, 4: 773-783).

According to various *in vitro and in vivo* study results on the anti-EGFR antibody, the anti-EGFR antibody inhibits cancer cell proliferation, reduces tumor-mediated angiogenesis, induces cancer cell apoptosis, and enhances the toxic effects of radiation therapy and traditional chemotherapy. As such, the anti-EGFR antibody can suppress tumors at various levels.

However, a liquid formulation comprising an anti-EGFR antibody for a therapeutic purpose may have problems in that protein multimers may be formed due to the aggregation property of the antibodies, and a deamination reaction may also take place due to proteolytic reactions. Such denaturation reaction may be caused by, for example, storing at an elevated temperature during transportation or a shear stress. If a liquid formulation comprising the anti-EGFR antibody shows aggregation due to denaturizaiton reactions, a precipitation and the formation of particles may take place, which may induce embolism.

In this regard, the liquid formulation may be subjected to filtration process before administration to a patient (e.g., injection by a syringe) in order to prevent aggregation. However, such additional step may render the administration method complicated and unsuitable for a clinical test. Also, the particle formation may continue to take place ever after the filtration process, leading to a decrease in stability.

Therefore, there has been a persistent need to develop a stable pharmaceutical formulation comprising an anti-EGFR antibody which has low turbidity, without showing aggregation or particle formation, even under a stress condition.

Meanwhile, a conventional pharmaceutical formulation comprising an anti-EGFR antibody normally comprises sodium chloride (NaCl) as an isotonization agent in order to reduce a pain reaction in the body caused by the osmotic pressure when the pharmaceutical formulation is administered. However, a pharmaceutical formulation comprising a protein therapeutics should contain appropriate ingredients which are suitable therefor. In this regard, the compatibility of NaCl should be also verified.

Disclosure of Invention

Technical Problem

An object of the present invention is to provide a pharmaceutical formulation comprising an anti-EGFR antibody which shows excellent stability.

Solution to Problem

The present invention provides a pharmaceutical formulation containing an anti-EGFR antibody, sodium acetate anhydrous, and polysorbate 80, which does not contain any sodium chloride (NaCl).

Advantageous Effects of Invention

Since a pharmaceutical formulation comprising an anti-EGFR antibody according to the present invention does not contain sodium chloride, unlike most of the conventional pharmaceutical formulations comprising an anti-EGFR antibody which contains sodium chloride, such pharmaceutical formulation has low turbidity, without showing aggregation or particle formation, even under accelerated conditions, and exhibits good stability. Therefore, the present pharmaceutical formulation can be effectively used for the treatment of cancer and the like.

Brief Description of Drawings

Figs. 1A, 1B and 1C respectively show the results of visual inspection, turbidity, and dynamic light scattering (DLS) analysis using the samples prepared for the selection of the type of a surfactant and a tonicifier.

Figs. 2A and 2B respectively show the results of turbidity and DLS analysis using the samples prepared for the selection of factors associated with aggregation and particle formation; and Figs. 3A and 3B respectively show the results of SE-HPLC and IE-HPLC analysis using these samples.

Figs. 4A, 4B and 4C respectively show the results of visual inspection, turbidity, and DLS analysis using the samples prepared for the screening of the type of a tonicifier.

Figs. 5A, 5B and 5C respectively show the results of visual inspection, turbidity, and DLS analysis using the samples prepared for the determination of the concentration of polysorbate 80.

Figs. 6A and 6B respectively show the results of turbidity and DLS analysis after storage at a high temperature using the pharmaceutical formulations of Preparation Examples and Comparative Preparation Examples.

Figs. 7A, 7B and 7C respectively show the results of turbidity changes of Preparation Examples 1 to 3 (formulations 3 to 5) at different temperatures.

Figs. 8A and 8B respectively show the SDS-PAGE results of the pharmaceutical formulations of Preparation Examples and Comparative Preparation Examples stored at 37°C, under non-reducing and reducing conditions.

Figs. 9 to 14 respectively show the results of visual inspection, protein concentration, SE-HPLC, IE-HPLC, IEF (Isoelectric focusing) and SDS-PAGE analysis of each sample prepared by Response Surface Methodology (RSM) design under the storage condition of 37°C for two weeks.

Figs. 15 to 18 respectively show the results of visual inspection, turbidity, DLS and potency 1 (binding assay) analysis of each sample prepared by RSM design under the storage condition of 60°C for 1 day.

Fig. 19 shows the result of the optimized plot of the samples prepared by RSM design.

Best Mode for Carrying Out the Invention

The present invention provides a liquid pharmaceutical formulation containing an anti-EGFR antibody, sodium acetate anhydrous, and polysorbate 80, which does not contain sodium chloride (NaCl).

The pharmaceutical formulation of the present invention may be preferably in the form of a liquid formulation in consideration of the user's convenience.

The pharmaceutical formulation of the present invention is characterized in that it contains no NaCl.

Generally, pharmaceutical formulations containing an anti-EGFR antibody comprise NaCl as tonicity modifier in order to reduce a pain reaction in the body caused by the osmotic pressure when the pharmaceutical formulation is administered. However, unlike conventional pharmaceutical formulations, NaCl is not comprised in the pharmaceutical formulations of the present invention so as to prepare a more stable pharmaceutical formulation comprising an anti-EGFR antibody which has low turbidity, without showing aggregation or particle formation, even under accelerated conditions. It is preferred that no NaCl is included in the anti-EGFR antibody formulation of the present invention, considering the compatibility with NaCl, which leads to minimal aggregation and particle formation, allowing the distribution of more stable products.

In one embodiment of the present invention, the main factors influencing turbidity, aggregation and particle formation of an antibody formulation were examined. The main factors influencing turbidity were found to be NaCl, the concentration of buffering agent and pH (Fig. 2A); and according to DLS analysis, the main factors influencing the reduction of the monomer intensity were found to be pH and NaCl (Fig. 2B). Further, according to SE-HPLC analysis, the main factors influencing the main peak reduction were found to be pH and NaCl (Fig. 3A); and according to IE-HPLC analysis, the main factors influencing the main peak reduction were pH and the concentration of buffering agent (Fig. 3B).

Based on these results, it is preferred that an antibody formulation should not contain NaCl so as to reduce turbidity and inhibit aggregation and particle formation.

The pharmaceutical formulation of the present invention contains an anti-EGFR antibody.

The anti-EGFR antibody can be comprised in a concentration of 1 to 16 mg/ml, preferably 2 to 10 mg/ml (for example, 10 mg/ml).

The anti-EGFR antibody for use in the present invention may be a conventional anti-EGFR antibody already known or commercially available. For example, an anti-EGFR antibody disclosed in U.S. Patent No. 6,217,866 or Korean Patent No. 1108642 can be used.

The pharmaceutical formulation of the present invention contains sodium acetate anhydrous as a buffering agent.

According to one embodiment of the present invention, the main factors influencing turbidity, aggregation and particle formation include the concentration of buffering agent and pH. Therefore, the type of buffering agent, and its concentration and pH are important factors.

Sodium acetate anhydrous is used to maintain the pH of a pharmaceutical formulation of the present invention, which minimizes the pH changes due to external influences.

Sodium acetate anhydrous can be replaced with a buffering agent selected from the group consisting of sodium phosphate, glutamate, histidine, and a combination thereof. However, in order to reduce turbidity and inhibit aggregation and particle formation in the pharmaceutical formulation, appropriate range of pH should be selected. According to the present invention, in or around the pH range of 5 to 6, it is preferred to use sodium acetate anhydrous as a buffering agent.

Sodium acetate anhydrous can be included in a concentration of 10 to 200 mM, preferably 10 to 100 mM (for example, 50 mM).

A pharmaceutical formulation of the present invention can have a pH ranging from 5 to 7, preferably 5.3 to 6.1.

The pharmaceutical formulation of the present invention contains polysorbate 80 as a surfactant.

Generally, a solution containing an antibody has a high surface tension in the air-water interface. To reduce such surface tension, antibodies have a tendency to aggregate in the air-water interface. By minimizing the antibody aggregation in the air-water interface, a surfactant helps maintain the biological activity of the antibodies in the solution.

According to one embodiment of the present invention, a mechanical stress test of an antibody formulation containing polysorbate 80, polysorbate 20 or poloxamer 188 as a surfactant was carried out. As a result, it was found that it is preferable to contain polysorbate 80 to achieve the effects of reducing turbidity and inhibiting aggregation and particle formation of the pharmaceutical formulation (*see* Example 1).

In addition, according to another embodiment of the present invention, with respect to an antibody formulation containing various concentration of polysorbate 80, an antibody formulation containing polysorbate 80 in a concentration of 0.05 to 2.0 mg/ml was found to have the effects of reducing turbidity and inhibiting aggregation and particle formation. In particular, the degree of aggregation and particle formation was found to be the least when polysorbate 80 was comprised in a concentration of 0.2 mg/ml (*see* Example 4).

Therefore, polysorbate 80 can be comprised in a concentration of 0.05 to 2.0 mg/ml, preferably 0.1 to 1.0 mg/ml (for example, 0.2 mg/ml).

The pharmaceutical formulation of the present invention can further contain a tonicifier.

A tonicifier in the present invention plays a role of inhibiting the aggregation and degradation of the pharmaceutical formulation.

In particular, it is preferable to employ mannitol, glycine or a combination thereof as a tonicifier, so as to reduce turbidity and inhibit aggregation and particle formation. According to one embodiment of the present invention, it was found that an antibody formulation containing mannitol or glycine as a tonicifier can significantly inhibit turbidity increase (*see* Example 3).

The tonicifier may be mannitol, which can be comprised in a concentration of 1 to 20% (w/v), preferably 2 to 10% (w/v), for example, 5% (w/v).

The tonicifier may be glycine, which can be comprised in a concentration of 1 to 10% (w/v), preferably 2 to 5% (w/v), for example, 3% (w/v).

The tonicifier may be a combination of mannitol and glycine wherein mannitol and glycine are mixed in a weight ratio of 5:1 to 1:5, which can be comprised in a concentration of 1 to 10% (w/v), preferably 2 to 5% (w/v).

One embodiment of the present invention provides a pharmaceutical formulation consisting of 10 mg/ml of an anti-EGFR antibody, 50 mM of sodium acetate anhydrous and 0.2 mg/ml of polysorbate 80, which does not contain NaCl.

Another embodiment of the present invention provides a pharmaceutical formulation consisting of 10 mg/ml of an anti-EGFR antibody, 50 mM of sodium acetate anhydrous, 0.2 mg/ml of polysorbate 80 and 5% (w/v) of mannitol, which does not contain NaCl.

A further embodiment of the present invention provides a pharmaceutical formulation consisting of 10 mg/ml of an anti-EGFR antibody, 50 mM of sodium acetate anhydrous, 0.2 mg/ml of polysorbate 80 and 3% (w/v) of glycine, which does not contain NaCl.

According to the stability analysis results examined under stress conditions, the pharmaceutical formulations of the present invention maintained colorless and transparent state, showing low rate of turbidity increase; and did not exhibit aggregations even after a long-term storage, maintaining good purity, as compared to a pharmaceutical formulation containing NaCl. Based on these results, it was found that a pharmaceutical formulation containing an anti-EGFR antibody has superior stability when no NaCl is included (*see* Test Examples).

A pharmaceutical formulation according to the present invention can further contain pharmaceutically acceptable additives such as a diluent, a carrier, a stabilizer, an antioxidant, a preservative, etc., if necessary.

The diluent can be a saline, glucose, Ringer or an aqueous buffer solution. The carrier can be a buffered saline solution, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol) and the like. The stabilizer can be an amino acid, cyclodextrin, polyethylene glycol, albumin (e.g., human serum albumin (HSA) and bovine serum albumin (BSA)), a salt (e.g., sodium chloride, magnesium chloride and calcium chloride), a chelator (e.g., EDTA) and the like. The antioxidant can be ascorbic acid, glutathione and the like. The preservative can be phenol, m-cresol, methyl-or propylparaben, chlorobutanol, benzalkonium chloride and the like.

A pharmaceutical formulation according to the present invention can be administered in various ways known in the art, for example, oral or parenteral administration. Examples of the parenteral administration may include intravenous, intramuscular, intraarterial, intramembranous, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, via bronchoscopy, subcutaneous, intra-subcutaneous layer, intraarticular, subcapsular, subarachnoidal, intrathecal, epidural and intrasternal injection and infusion, but are not limited thereto.

The daily dosage of a pharmaceutical formulation of the present invention may range from 0.01 to 10 mg/kg (body weight), preferably 0.01 to 1.0 mg/kg body weight, and it can be administered once or in divided doses per day. However, the actual dose of the pharmaceutical formulation should be determined in consideration of various relevant factors such as the route of administration, age, sex, weight, disease severity, etc., and thus, the above dosage does not limit the scope of the present invention in any way.

Examples

Hereinafter, the present invention is explained in detail by Examples. The following Examples are intended to further illustrate the present invention without limiting its scope.

The providers of the reagents used in the Examples below are as follows.

<u>Reagents and test solutions</u>

Sodium acetate anhydrous (Scharlau, Cat. No. SO0032)

Sodium phosphate monobasic (Scharlau, Cat. No. SO0333)

Sodium phosphate dibasic (JT Baker, Cat. No. 3817-05)

Polysorbate 20 (Tween 20, Merck, Cat. No. 8.17072.1000)

Polysorbate 80 (Tween 80, Fluka, Cat. No. 59924)

Poloxamer 188 (Pluronic F68, Sigma, Cat. No. P-1300)

NaCl (Scharlau, Cat. No. SO0225)

Mannitol (JT Baker, Cat. No. 238506)

Sorbitol (Sigma, Cat. No. S7547)

Glycine (Sigma, Cat. No. 15527)

Trehalose dihydrate (Sigma, Cat. No. T5251)

Arginine hydrochloride (Arg-HCl, Scharlau, Cat. No. AR0125)

The samples in the Examples below were prepared by mixing the ingredients according to each design condition, filtering them through a 0.22 µm filter, subdividing them into 3 ml glass vials in a clean bench, and stoppering them with a rubber plug, which were stored for use in the experiments.

Test methods used in the present invention are as follows.

(1) Mechanical stress: A vial was fixed in a vortex mixer (Vortex-Genie 2, Scientific Industries) and vortexed at 3000 rpm at room temperature for 4 hours.

(2) Thermal stress: A vial was put into a 37 °C thermo-hygrostat (LHD-2250C, Labtech) or 60 °C thermostat (DX7, Hanyoung).

(3) Visual inspection: Aggregation formation was observed with naked eyes.

(4) Turbidity: After preparing 1 mL of each sample solution without dilution, the absorbance was measured using a UV spectrophotometer (LibraS32, Biochem) at 350 nm, to analyze turbidity.

(5) Dynamic light scattering (DLS): After preparing at least 1 mL of each sample solution without dilution, the size distribution chart of the sample solution was analyzed using a DLS instrument (Zetasizer Nano ZS90, Malvern Instrument).

(6) SDS-PAGE: Analysis was carried out by a conventional SDS-PAGE analysis method using an electrophoresis device.

(7) Isoelectric focusing (IEF): Analysis was carried out by a conventional IEF analysis method using an electrophoresis device.

(8) UV protein quantitation: Absorbance was measured using a spectrophotometer at 280 nm and protein quantitation analysis was carried out.

(9) SE-HPLC: Analysis was carried out by using SE-HPLC analytical column (TSK gel G3000SW$_{XL}$, Cat. No. 08541, Tosoh Corporation).

(10) IE-HPLC: Analysis was carried out by using IE-HPLC analytical column (Propac WCX-10 analytical, Cat. No. 054993, Dionex).

(11) Potency 1 (Binding assay): ELISA reader (Spectra Max 190, Molecular Devices) was used for potency 1 analysis.

(12) Potency 2 (Cell based assay): The identical number of cells calculated by cell counting were alliquoted into each well of a 96-well plate. After culturing them for 24 hours, culture medium was removed. Then, 100 μL of diluted sample solution was put into each well. And 100 μL of diluted standard substance and diluted sample were put into each well and cultured for 5 days. 40 μL of MTS reagent was put into each well and was wrapped by a foil and reacted at 37°C for 3 hours. The absorbance was measured at 490 nm using ELISA reader (Spectra Max 190, Molecular Devices).

<Screening for the preparation of optimized antibody formulation>

Example 1: Selection of type of surfactant and tonicifier

To select the type of surfactant and tonicifier which can inhibit aggregation and particle formation, each sample was prepared by mixing the ingredients as shown in Table 1.

[Table 1]

No	Buffer	pH	Surfactant	Tonicifier
1	50mM Na-Acetate	5.8	—	110 mM NaCl

2	50 mM Na-Acetate	5.8	2 mg/ml PS80	110 mM NaCl
3	50 mM Na-Acetate	5.8	2 mg/ml PS20	110 mM NaCl
4	50 mM Na-Acetate	5.8	2 mg/ml P-188	110 mM NaCl
5	50 mM Na-Acetate	5.8	—	110 mM NaCl / 3% Gly
6	50 mM Na-Acetate	5.8	—	110 mM NaCl / 3% Arg-HCl
7	50 mM Na-Acetate	5.8	—	110 mM NaCl / 6% Mannitol
8	50 mM Na-Acetate	5.8	—	110 mM NaCl / 6% Sorbitol
9	50 mM Na-Acetate	5.8	—	110 mM NaCl / 6% Trehalose

A mechanical stress test was performed with the samples prepared above, and the results of visual inspection, turbidity, and DLS analysis are shown in Figs. 1A, 1B, and 1C, respectively.

As shown in Fig. 1, turbidity determined by visual inspection was in the order of #1, #5, #8 > #7, #9 > #6 > #2, #3, #4. And, the measurement of turbidity of the samples revealed the following results: the samples with a surfactant didn't show turbidity increase; and the samples with an additional tonicifier showed turbidity decrease. More particularly, Arg-HCl-added sample (#6) showed 46% decrease, and trehalose-added sample (#9) showed 12% decrease in turbidity as compared to sample #1. DLS analysis revealed that a surfactant inhibited aggregation formation, while a tonicifier increased aggregation intensity.

Therefore, with regard to a surfactant (considering turbidity, aggregation formation and other competing drug formulations), polysorbate 80 (PS80) was preferred over polysorbate 20 (PS20) or poloxamer 188 (P-188); and with regard to a tonicifier, Arg-HCl was preferred, in terms of turbidity.

Example 2: Selection of factors for aggregation and particle formation

To identify the main factors influencing aggregation and particle formation in a pharmaceutical formulation containing an anti-EGFR antibody (GC1118A, Mogam Biotechnology Institute), each sample was prepared by mixing the ingredients as shown in Table 2.

[Table 2]

No	Protein concentration (mg/ml)	Buffer	pH	Surfactant	Tonicifier
1	2	10 mM Na-Acetate	4	—	—
2	2	10 mM Na-Phosphate	8	2 mg/ml PS80	—
3	2	100 mM Na-Acetate	4	2 mg/ml PS80	3% Arg-HCl
4	2	100 mM Na-Phosphate	8	—	3% Arg-HCl
5	10	10 mM Na-Acetate	4	2 mg/ml PS80	3% Arg-HCl
6	10	10 mM Na-Phosphate	8	—	3% Arg-HCl
7	10	100 mM Na-Acetate	4	—	—
8	10	100 mM Na-Phosphate	8	2 mg/ml PS80	—
9	2	10 mM Na-Acetate	4	2 mg/ml PS80	300 mM NaCl/3% Arg-HCl
10	2	10 mM Na-Phosphate	8	—	300 mM NaCl/3% Arg-HCl
11	2	100 mM Na-Acetate	4	2 mg/ml PS80	300 mM NaCl
12	2	100 mM Na-Phosphate	8	—	300 mM NaCl
13	10	10 mM Na-Acetate	4	2 mg/ml PS80	300 mM NaCl
14	10	10 mM Na-Phosphate	8	—	300 mM NaCl
15	10	100 mM Na-Acetate	4	—	300 mM NaCl/3% Arg-HCl
16	10	100 mM Na-Phosphate	8	2 mg/ml PS80	300 mM NaCl/3% Arg-HCl

A thermal stress test was performed with the samples prepared above. After the thermal stress test (storage at 37°C for 2 weeks), the turbidity analysis by visual inspection, and DLS, SE-HPLC and IE-HPLC analyses were conducted to select the three main factors influencing aggregation and particle formation.

For the selection of the main factors, Minitab software was used in such a manner as to analyze the results from the pareto chart (alpha=0.15). The results are shown in Figs. 2A and 2B, and Figs. 3A and 3B.

As shown in Fig. 2, the main factors influencing turbidity were NaCl, buffer concentration and pH (Fig. 2A). Also, DLS analysis results indicated that the main factors influencing the reduction of monomer intensity were pH and NaCl (Fig. 2B).

In addition, as shown in Fig. 3, the SE-HPLC analysis results indicated that the main factors influencing the main peak reduction were pH and NaCl (Fig. 3A). Also, IE-HPLC analysis results indicated that the main factors influencing the main peak reduction were pH and buffer concentration (Fig. 3B).

Based on the results, pH, NaCl and buffer concentration were selected as the main factors influencing aggregation and particle formation. In the following experiments, Response Surface Methodology (RSM) design was conducted using Minitab program.

Example 3: Selection of conditions for pharmaceutical formulation by RSM design

In order to examine the main factors influencing aggregation and particle formation selected in Example 2 with regard to an optimized condition for a stable liquid formulation under a thermal stress, RSM design was conducted by Minitab program (CCI, level 5), and various samples were prepared in accordance with to the pH (4-8), and concentrations of NaCl (0-300 mM) and buffer (10-100mM).

Specific ingredients of the samples prepared are described in Table 3 below.

[Table 3]

No	Protein concentration (mg/ml)	Buffer	pH	Tonicifier
1	10 mg/ml	28mM Na-Acetate	4.8	61mM NaCl
2		28mM Na-Phosphate	7.2	61mM NaCl
3		82mM Na-Acetate	4.8	61mM NaCl
4		82mM Na-Phosphate	7.2	61mM NaCl
5		28mM Na-Acetate	4.8	239mM NaCl
6		28mM Na-Phosphate	7.2	239mM NaCl
7		82mM Na-Acetate	4.8	239mM NaCl
8		82mM Na-Phosphate	7.2	239mM NaCl
9		55mM Na-Acetate	4.0	150 mM NaCl
10		55mM Na-Phosphate	8.0	150 mM NaCl
11		10 mM Na-Acetate	6.0	150 mM NaCl
12		100 mM Na-Acetate	6.0	150 mM NaCl
13		55mM Na-Acetate	6.0	—
14		55mM Na-Acetate	6.0	300 mM NaCl
15		55mM Na-Acetate	6.0	150 mM NaCl
16		55mM Na-Acetate	6.0	150 mM NaCl
17		55mM Na-Acetate	6.0	150 mM NaCl
18		55mM Na-Acetate	6.0	150 mM NaCl
19		55mM Na-Acetate	6.0	150 mM NaCl
20		55mM Na-Acetate	6.0	150 mM NaCl

First, each sample was prepared by mixing the ingredients as indicated in Table 3. The prepared samples were subjected to thermal tests, under the storage condition of: (i) 37 °C for two weeks; or (ii) 60 °C for one day.

The results of visual inspection, protein concentration, SE-HPLC, IE-HPLC, IEF and SDS-PAGE analyses of each sample under the storage condition of 37 °C for two weeks are shown in Figs. 9-14. Visual inspection analysis result revealed that only #9 was opaque while all others were colorless and transparent, and IEF results revealed that #9 showed band dragging phenomenon caused by particle formation.

The results of visual inspection, turbidity and DLS analysis, and binding assay 1 of each sample under the storage condition of 60 °C for one day are shown in Figs. 15-18. Visual inspection analysis showed that turbidity was in the order of #9 > #5 > #7 > #1 and #3 (all other samples were colorless and transparent), and the binding assay 1 result showed that #15 sample had EC_{50}% of 114 to121% as compared to a control group.

In addition, an optimized plot based on the above results is shown in Fig. 19 and Table 4.

[Table 4]

	37 °C, 2 weeks	60 °C, 1 day
Turbidity	pH (p=0.004), R^2=0.4749	pH, NaCl (p=0.001), R^2=0.8296
DLS	pH (p=0.001), R^2=0.5482	pH, NaCl (p<0.001), R^2=0.9286
Protein concentration	No concentration change as compared to initial (±4% range)	—
SE-HPLC	pH (p<0.001), R^2=0.6019	—
IE-HPLC	pH, NaCl (P<0.001), R^2=0.9486	—
SDS-PAGE	pH (p<0.001), R^2=0.6257	—
IEF	No band pattern change as compared to initial (except #9, particle formation)	—
Binding Assay	pH, Buffer (p=0.116), R^2=0.3715	pH, NaCl (p<0.001), R^2=0.8506
Cell based Assay	—	—

The conditions of a preferred pharmaceutical preparation suggested by the statistical analysis based on the results of Table 4 are shown in Table 5 below.

[Table 5]

	Protein concentration	Buffer solution	pH	Tonicifier

Optimized plot base	10 mg/ml	10-94 mM Na-Acetate	5.94	no NaCl
Contour base	10 mg/ml	10-100 mM Na-Acetate	5.3-6.1	0 mM NaCl

More particularly, the condition satisfying a desired potency while minimizing aggregation, particle formation, and potential variation under the thermal stress condition was found to be protein (anti-EGFR antibody) concentration of 10 mg/ml, 10 to 100 mM sodium acetate, pH of 5.3 to 6.1, and 0 mM NaCl (based on contour). Also, the optimum safety condition among the above conditions was pH of 5.94, 50 mM sodium acetate, and no NaCl (i.e., 0 mM NaCl) (based on optimized plot).

Example 4: Screening of the type of tonicifier

To select the type of a tonicifer to be used in the pharmaceutical formulation according to the present invention, each sample was prepared by mixing the ingredients as shown in Table 6. The prepared samples were stored at 60 °C for one day, and then the results of visual inspection, turbidity, and DLS analysis are shown in Figs. 4A, 4B, and 4C, respectively.

[Table 6]

No	Protein concentration	Buffer	pH	Tonicifier	Note
1	10 mg/ml	50 mM Na-Acetate	5.8	—	Control
2	10 mg/ml	50 mM Na-Acetate	5.8	110 mM NaCl	Current temporary formulation
3	10 mg/ml	50 mM Na-Acetate	5.8	3% Glycine	Tonicifier change (Isotonic level)
4		50 mM Na-Acetate	5.8	3% Arg-HCl	
5		50 mM Na-Acetate	5.8	5% Mannitol	
6		50 mM Na-Acetate	5.8	5% Sorbitol	
7		50 mM Na-Acetate	5.8	5% Trehalose	

As shown in Fig. 4, visual inspection analysis result showed that all samples were colorless and transparent (Fig. 4A), and turbidity and DLS analysis results showed that the formulations containing 3% glycine or 5% mannitol significantly inhibited the increase in turbidity (Figs. 4B and 4C).

Example 5: Determination of concentration of polysorbate 80

To determine the concentration of polysorbate 80 to be used in a pharmaceutical formulation according to the present invention, each sample was prepared by mixing the ingredients as shown in Table 7. Then the prepared samples were subjected to mechanical tests, and the results of visual inspection, turbidity, and DLS analysis are shown in Figs. 5A, 5B, and 5C, respectively.

[Table 7]

No	Protein concentration (mg/ml)	Buffer	pH	Surfactant	Tonicifier
1	12.60	50 mM Na-Acetate	5.8	-	110 mM NaCl
2				0.05 mg/ml Polysorbate 80	
3				0.1 mg/ml Polysorbate 80	
4				0.2 mg/ml Polysorbate 80	
5				0.5 mg/ml Polysorbate 80	
6				1.0 mg/ml Polysorbate 80	
7				2.0 mg/ml Polysorbate 80	

As shown in Fig. 5, only sample #1 was opaque and all other samples were colorless and transparent (Fig. 5A). In addition, there was no significant turbidity change except in sample #1, and turbidity inhibition effect was observed throughout the concentration range of 0.05 to 2.0 mg/ml (Fig. 5B). Meanwhile, DLS analysis result also revealed that the aggregation inhibition effect was observed throughout the concentration range of 0.05 to 2.0 mg/ml (Fig. 5C).

Among them, 0.2 mg/ml of polysorbate 80 resulted in the lowest aggregation and was chosen as the optimum concentration.

Preparation Examples 1 to 3 and Comparative Preparation Examples 1 and 2:

Preparation of pharmaceutical formulations

Pharmaceutical liquid formulations were prepared in a conventional manner using the ingredients as shown in Table 8.

[Table 8]

<table>
<tr><th></th><th>Protein concentration</th><th>Buffer</th><th>pH</th><th>Surfactant</th><th>Tonicifier</th></tr>
<tr><td>Comparative Preparation Example 1 (Temporary formulation #1)</td><td rowspan="2">10.0 mg/ml</td><td rowspan="2">50 mM Na-Acetate</td><td rowspan="2">5.8</td><td>-</td><td>110 mM NaCl</td></tr>
<tr><td>Comparative Preparation Example 2 (Temporary formulation #2)</td><td>0.2 mg/ml polysorbate 80 (PS80)</td><td>110 mM NaCl</td></tr>
<tr><td>Preparation Example 1 (#3, no NaCl and PS80)</td><td rowspan="3">10.0 mg/ml</td><td rowspan="3">50 mM Na-Acetate</td><td rowspan="3">5.7 (±0.4)</td><td>0.2 mg/ml PS80</td><td>no NaCl</td></tr>
<tr><td>Preparation Example 2 (#4, Glycine and PS80)</td><td>0.2 mg/ml PS80</td><td>3% Glycine</td></tr>
<tr><td>Preparation Example 3 (#5, Mannitol and PS80)</td><td>0.2 mg/ml PS80</td><td>5% Mannitol</td></tr>
</table>

Test Example: Stability analysis of pharmaceutical formulations

After storage of the pharmaceutical formulations prepared in accordance with Preparation Examples 1 to 3 and Comparative Preparation Examples 1 and 2 under the stress conditions described below, visual inspection, turbidity, DLS, protein content, SE-HPLC, IE-HPLC, SDS- PAGE, IEF and potencies 1 and 2 analyses were conducted.

Stress conditions

(1) storage at 60 °C for 2 weeks (1 day, 3 days, 1 week and 2 weeks)

(2) storage at 5 °C, 25 °C or 37 °C for 2 weeks to 12 months (initial, 2 weeks, 4 weeks, 6 weeks, 8 weeks, and 3 to 12 months)

Results

Under the condition of storage at 60 °C for two weeks, visual inspection analysis results revealed that the pharmaceutical formulations of Comparative Preparation

Examples 1 and 2 became opaque, whereas the pharmaceutical formulation of Preparation Example 3 remained colorless and transparent after 2-week storage. Turbidity and DLS analysis indicated that the pharmaceutical formulation of Preparation Example 3, which contained mannitol and polysorbate 80, showed the smallest increase in turbidity (Figs. 6A and 6B).

Under the condition of storage at 5 °C, 25 °C or 37 °C for 2 weeks to 12 months, visual inspection analysis result revealed that all pharmaceutical formulations were colorless and transparent under any of the storage conditions. Turbidity change analysis of the formulations stored at different temperatures indicated that the samples stored at 5 °C or 25 °C showed no change in turbidity regardless of the type of the formulation. However, among the samples stored at 37 °C, formulation #4 of Preparation Example 2 showed a 2.3-fold increase in turbidity after 8-week storage as compared to the initial point (Fig. 7).

On the other hand, the UV protein quantitative analysis revealed that all formulations under any of the temperature conditions were in the range of 95 to 105% as compared to the initial point, showing no significant changes.

The SDS-PAGE analysis result indicated that there was no difference in the band patterns according to storage temperatures between the Preparation Examples and Comparative Preparation Examples. However, as can be seen from Figs. 8A and 8B, the results at initial point and after 8-week storage at 37°C under non-reducing and reducing conditions indicated differences in the band patterns between the samples at the initial point and after the high temperature storage. Under the non-reducing condition, all of the formulations #1 to #5 of the Preparation Examples and Comparative Preparation Examples after storage at 37°C showed increase in the band intensity at 250 kD, and 37 to 50 kD as compared to the initial point. Also, under the reducing condition, the formulations #1 to #5 of the Preparation Examples and Comparative Preparation Examples after storage at 37°C showed increase in the band intensity at 75 kD, and new band formations at 25 to 50 kD, as compared to the initial point.

Meanwhile, IEF results indicated that there was no difference in IEF band patterns between the formulations or according to different temperatures under any of the storage conditions, and the bands were located between the pI of 7.8 to 8.3.

SE-HPLC analysis results indicated that all of the formulations #1 to #5 of the Preparation Examples and Comparative Preparation Examples showed no significant purity change after storage at 5 °C or 25 °C. However, after storage at 37 °C for 8 weeks, formulations #1, #2, #3, #4 and #5 showed the values of 95.66%, 95.15%, 96.46%, 96.42% and 96.56%, respectively, which implies purity decreases of 3.14 to 4.55% as compared to the initial point. As such, it was found that the formulations of Preparation Examples of the present invention showed significantly higher stability as compared to the Comparative Preparation Examples. Particularly, the formulations of Comparative Preparation Examples showed large amount of purity decrease, which was caused by the increase of pre-peak portion (presumed to be aggregations) preceding the main peak.

The potency 2 analysis (cell-based assay) results revealed that all the formulations, regardless of the storage temperature, showed EC_{50} of 70 to 130% as compared to the control group, indicating that there was no significant change.

The above results indicates that a tonicifier for inhibiting the aggregation and particle formation under the heat stress condition for use in a pharmaceutical formulation comprising an anti-EGFR antibody may be NaCl, glycine, mannitol, or the like, preferably, mannitol or no NaCl; and the preferred concentration of polysorbate 80 for inhibiting the aggregation and particle formation under the mechanical stress condition is 0.2 mg/ml.

Also, the result of stability analysis of the pharmaceutical formulations after storage at the high temperature of 60 °C indicated that a formulation comprising 10 mg/ml an anti-EGFR antibody can be stabilized under the mechanical/thermal stress by containing 50 mM sodium acetate, pH of 5.7±0.4, 0.2 mg/ml polysorbate 80 and 5% mannitol.

In addition, the result of stability analysis of the pharmaceutical formulations after storage at 5°C, 25°C and 37°C for 8 weeks indicated that it is preferable for the formulation comprising 10 mg/ml anti-EGFR antibody to further contain 50 mM sodium acetate, pH 5.7±0.4, 0.2 mg/ml polysorbate 80, without containing NaCl in terms of physical/chemical stability.

Claims:

[Claim 1]

A pharmaceutical formulation comprising an anti-epidermal growth factor receptor (EGFR) antibody, sodium acetate anhydrous, and polysorbate 80, wherein no sodium chloride (NaCl) is included in the pharmaceutical formulation.

[Claim 2]

The pharmaceutical formulation of claim 1, which further comprises a tonicifier selected from mannitol, glycine or a combination thereof.

[Claim 3]

The pharmaceutical formulation of claim 1, wherein the pH of the pharmaceutical formulation ranges from pH 5.3 to 6.1.

[Claim 4]

The pharmaceutical formulation of claim 1, wherein the anti-EGFR antibody is comprised in a concentration of 2 to 10 mg/ml.

[Claim 5]

The pharmaceutical formulation of claim 1, wherein the sodium acetate anhydrous is comprised in a concentration of 10 to 100 mM.

[Claim 6]

The pharmaceutical formulation of claim 1, wherein the polysorbate 80 is comprised in a concentration of 0.05 to 2.0 mg/ml.

[Claim 7]

The pharmaceutical formulation of claim 2, wherein the tonicifier is mannitol and is comprised in a concentration of 1 to 20% (w/v).

[Claim 8]

The pharmaceutical formulation of claim 2, wherein the tonicifier is glycine and is comprised in a concentration of 1 to 10% (w/v).

[Claim 9]

The pharmaceutical formulation of claim 2, wherein the tonicifier is a mixture of mannitol and glycine which are mixed in a weight ratio of 5:1 to 1:5, and is comprised in a concentration of 1 to 10% (w/v).

Fig. 1A

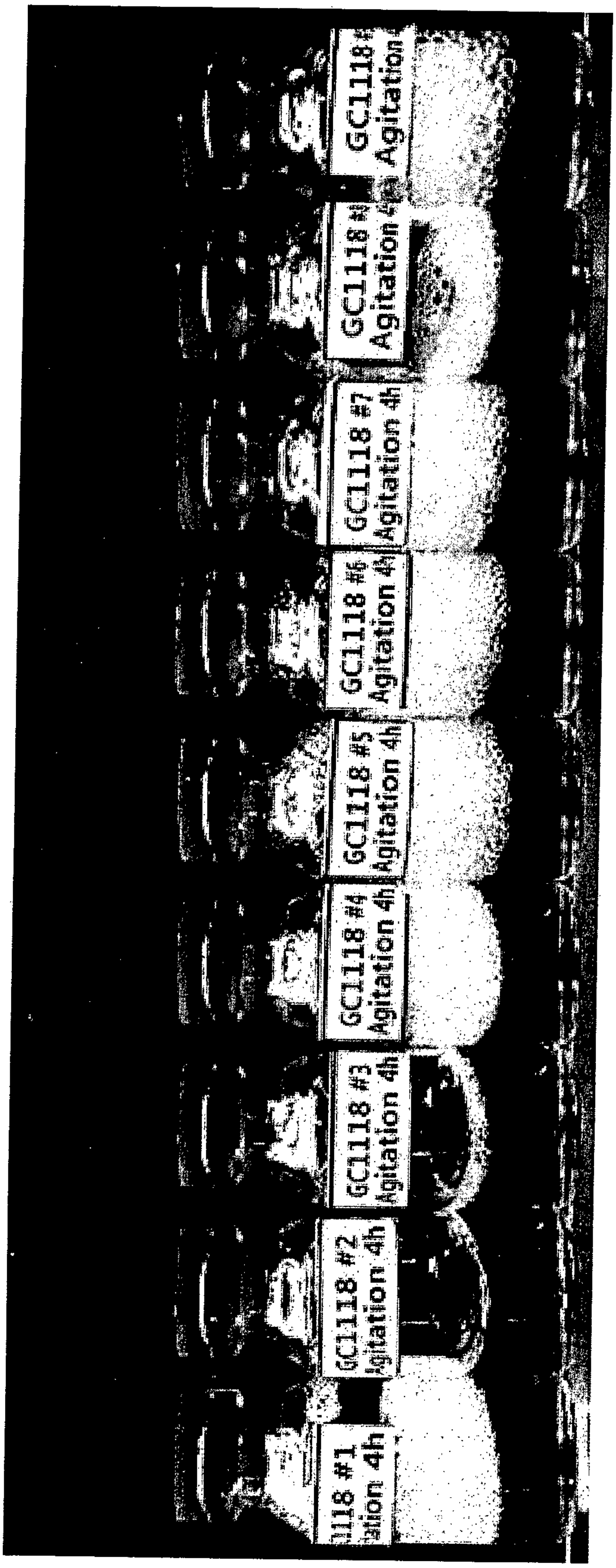

118 #1
ation 4h
GC1118 #2
Agitation 4h
GC1118 #3
Agitation 4h
GC1118 #4
Agitation 4h
GC1118 #5
Agitation 4h
GC1118 #6
Agitation 4h
GC1118 #7
Agitation 4h
GC1118 #
Agitation 4
GC1118 #
Agitation

Fig. 1B

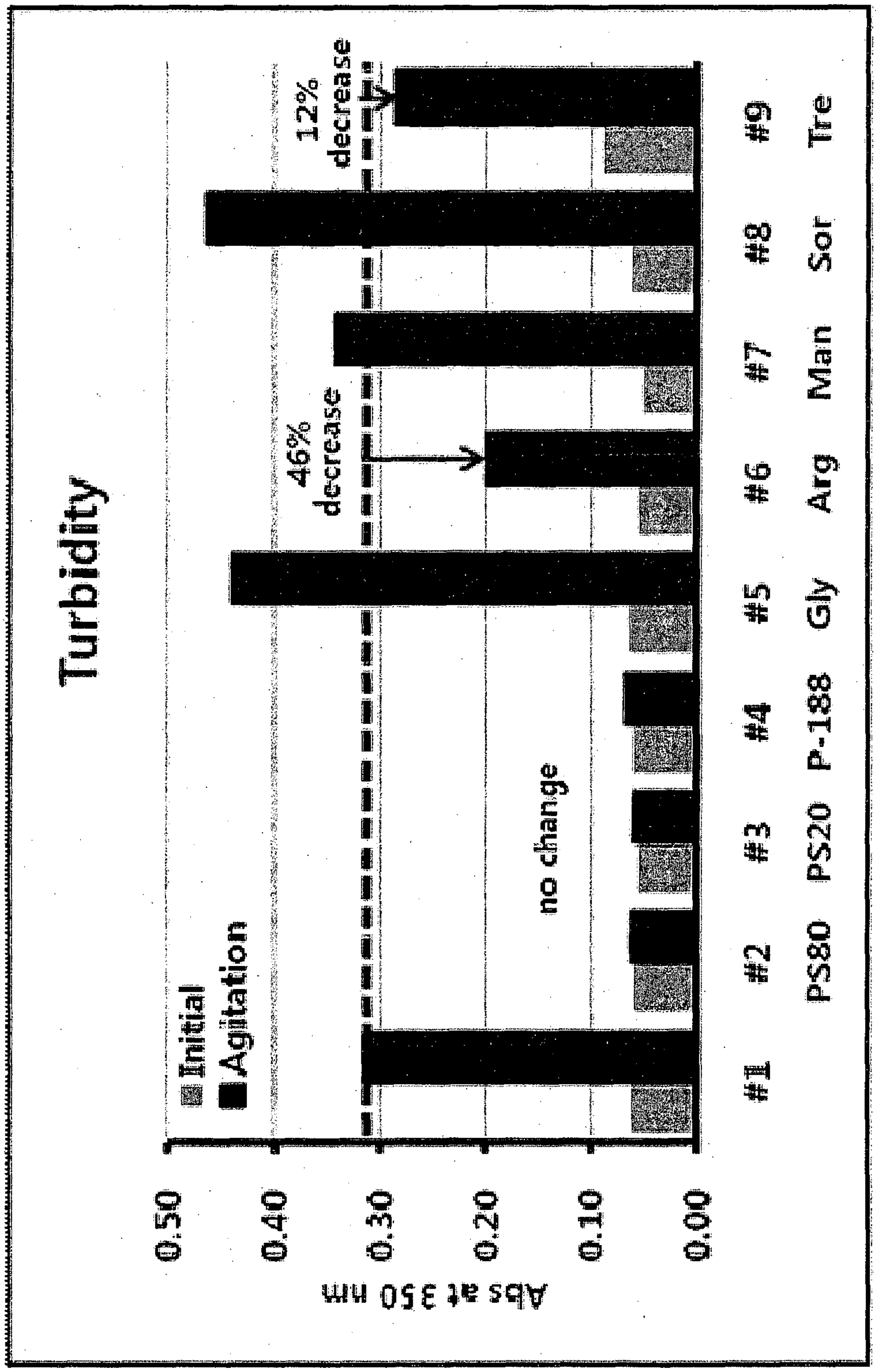
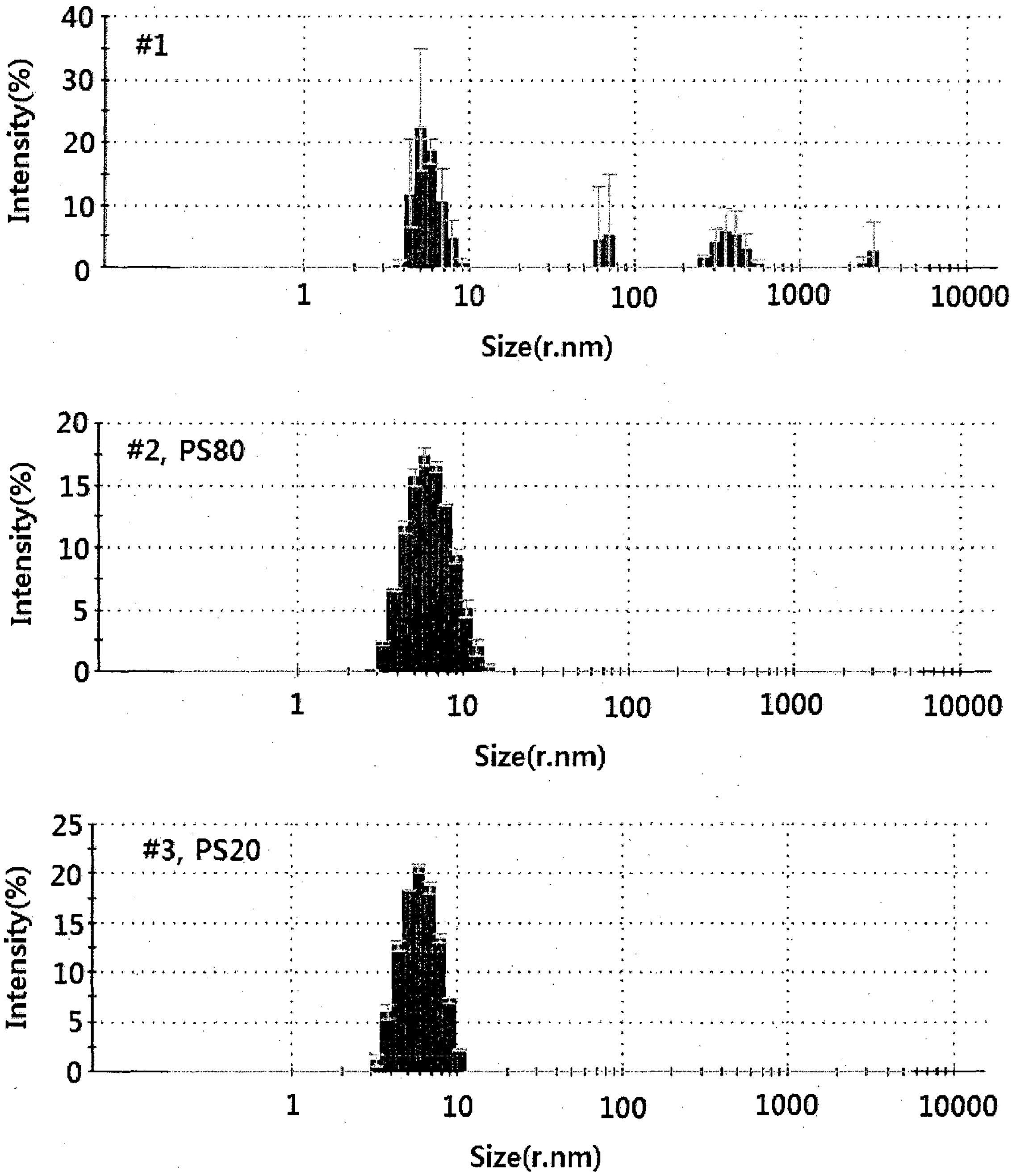

Turbidity
Initial
Agitation
Abs at 350 nm
0.50
0.40
0.30
0.20
0.10
0.00
no change
46% decrease
12% decrease
#1
#2 PS80
#3 PS20
#4 P-188
#5 Gly
#6 Arg
#7 Man
#8 Sor
#9 Tre

Fig. 1C



#1
Intensity(%)
40
30
20
10
0
1
10
100
1000
10000
Size(r.nm)
#2, PS80
Intensity(%)
20
15
10
5
0
1
10
100
1000
10000
Size(r.nm)
#3, PS20
Intensity(%)
25
20
15
10
5
0
1
10
100
1000
10000
Size(r.nm)

Fig.1C (Continued)

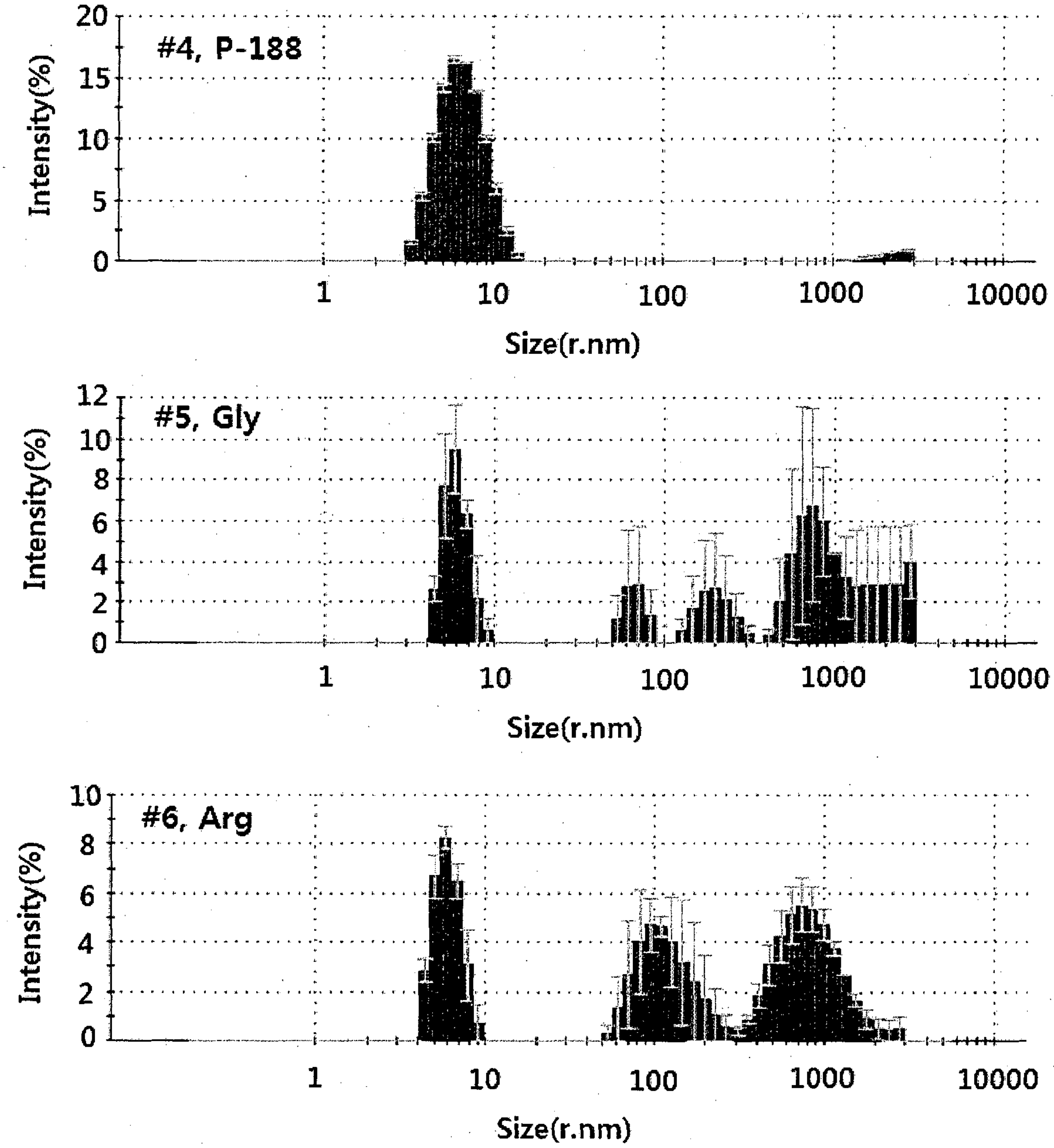


Fig.1C (Continued)

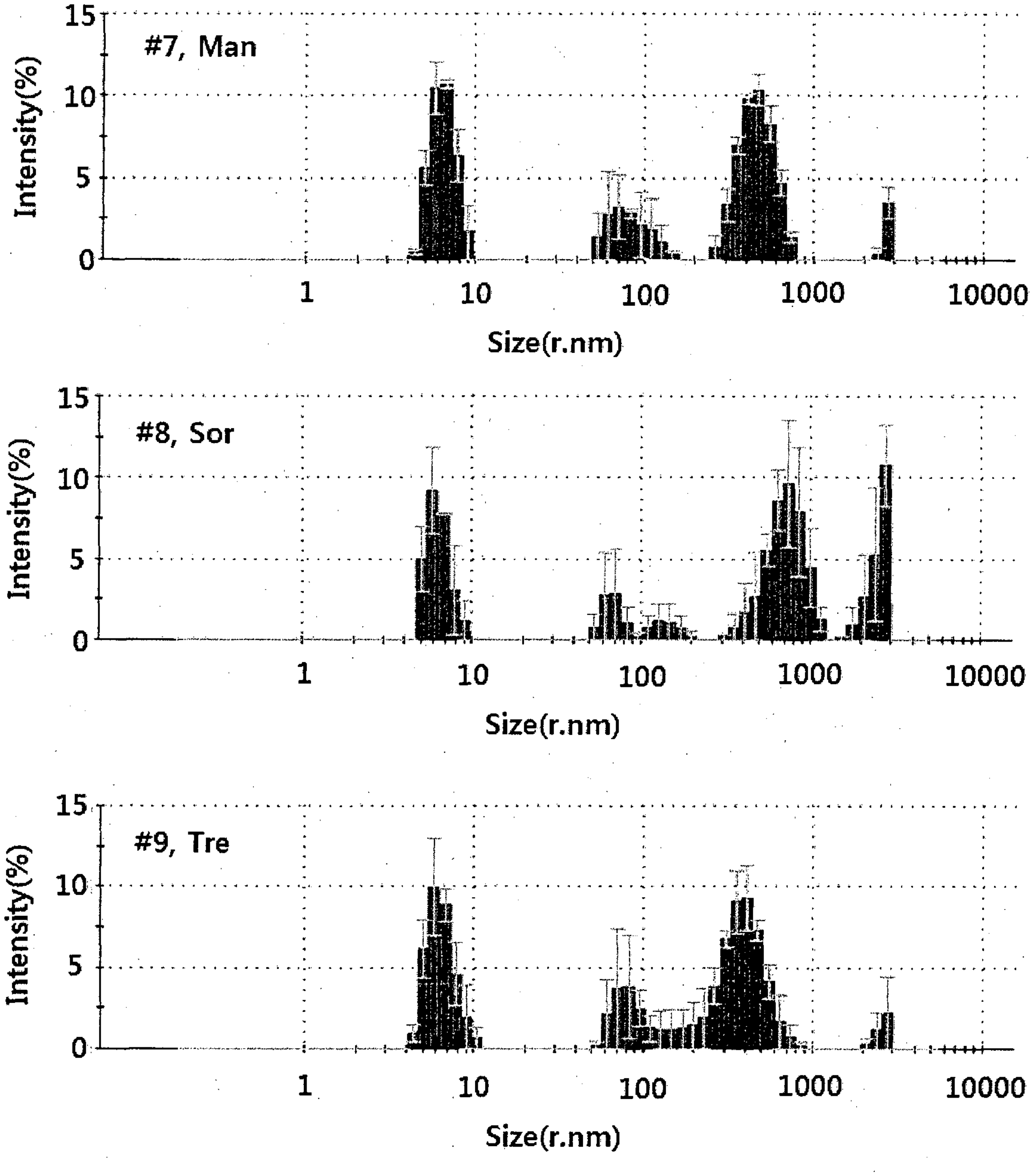


Fig. 2A

	Normalized Turbidity (Initial)	Normalized Turbidity (37°C/2W)	Normalized Turbidity (amount of change)
GC1118 #1	0.0045	0.0050	0.0005
GC1118 #2	0.0083	0.0093	0.0010
GC1118 #3	0.0110	0.0145	0.0035
GC1118 #4	0.0074	0.0133	0.0058
GC1118 #5	0.0051	0.0061	0.0010
GC1118 #6	0.0050	0.0064	0.0014
GC1118 #7	0.0041	0.0048	0.0007
GC1118 #8	0.0054	0.0058	0.0004
GC1118 #9	0.0072	0.0124	0.0052
GC1118 #10	0.0111	0.0127	0.0015
GC1118 #11	0.0075	0.0306	0.0231
GC1118 #12	0.0052	0.0094	0.0042
GC1118 #13	0.0051	0.0102	0.0052
GC1118 #14	0.0047	0.0059	0.0011
GC1118 #15	0.0049	0.0413	0.0364
GC1118 #16	0.0055	0.0074	0.0020

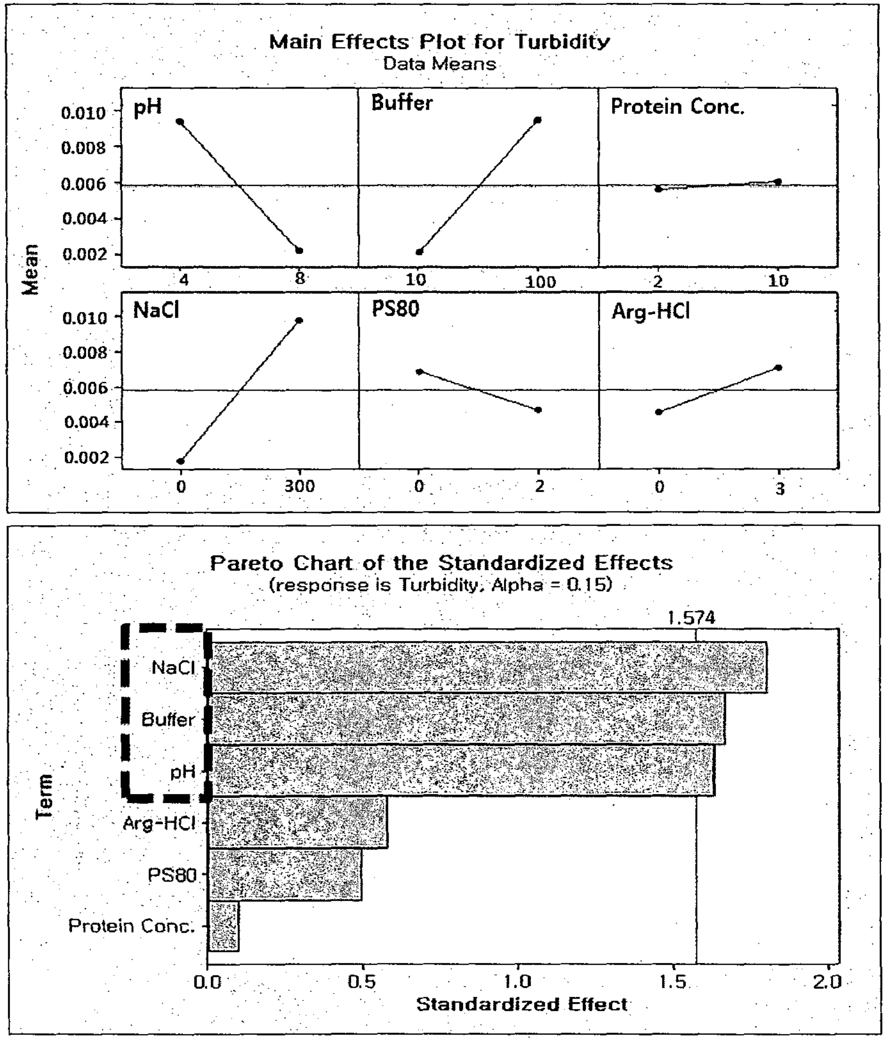


Fig. 2B

	Monomer Intensity% (≤ 11nm) (Initial)	Monomer Intensity% (≤ 11nm) (37°C/2W)	Intensity (compared to Initial, %)
GC1118 #1	100.0	100.0	100.0
GC1118 #2	100.0	100.0	100.0
GC1118 #3	97.5	60.7	62.3
GC1118 #4	97.7	99.6	101.9
GC1118 #5	100.0	91.7	91.7
GC1118 #6	100.0	100.0	100.0
GC1118 #7	100.0	100.0	100.0
GC1118 #8	100.0	100.0	100.0
GC1118 #9	97.6	38.9	39.9
GC1118 #10	100.0	99.3	99.3
GC1118 #11	100.0	14.0	14.0
GC1118 #12	100.0	96.1	96.1
GC1118 #13	100.0	35.7	35.7
GC1118 #14	100.0	100.0	100.0
GC1118 #15	99.3	0.0	0.0
GC1118 #16	100.0	99.3	99.3

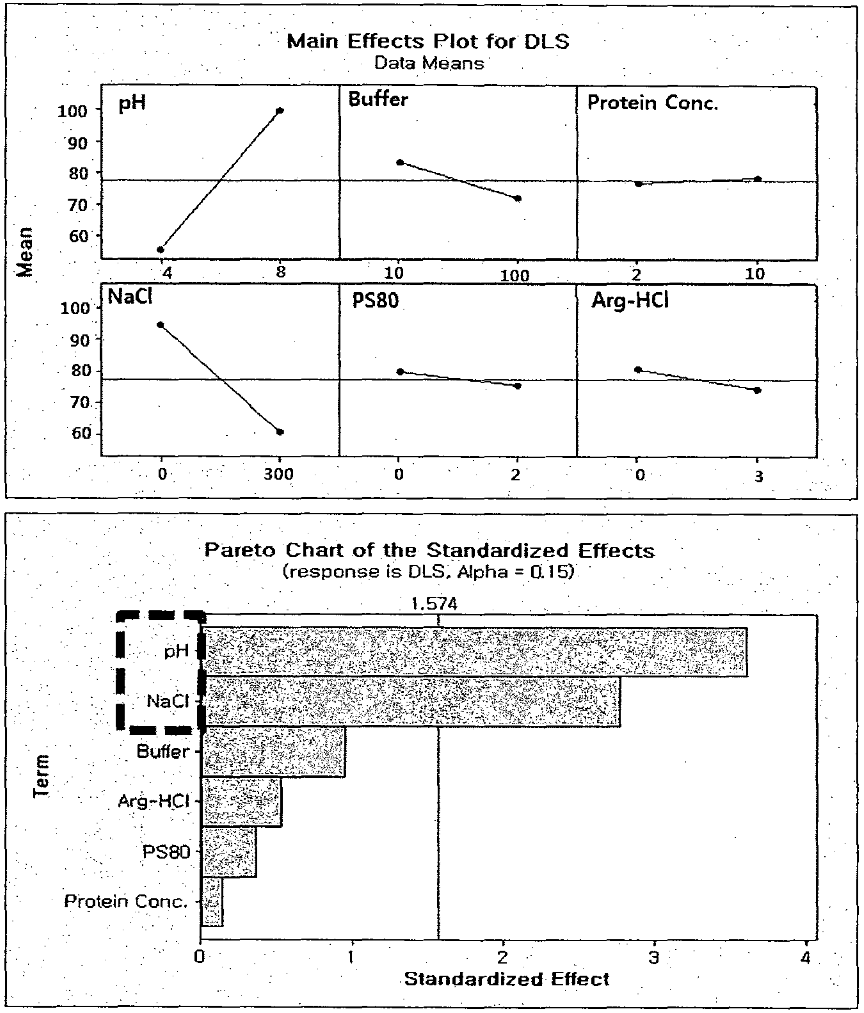
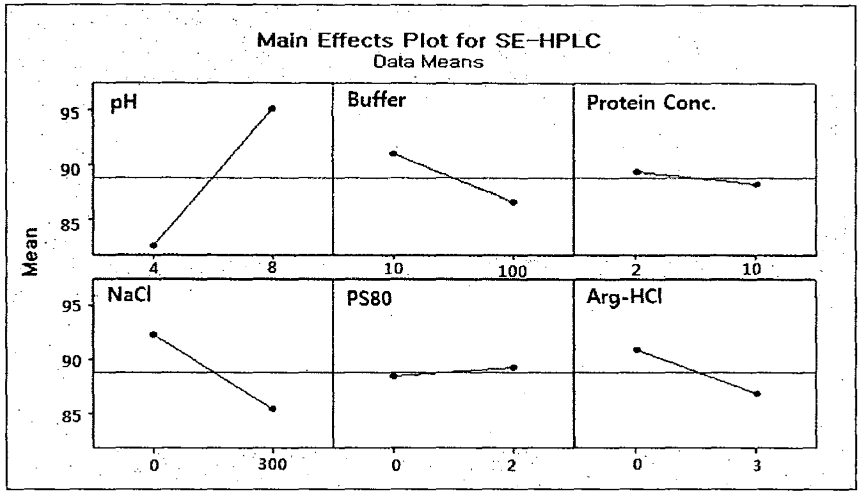


Fig. 3A

	Main Area% (Initial)	Main Area% (37°C/2W)	Main Area (compared to initial, %)
GC1118 #1	99.89	94.16	94.3
GC1118 #2	99.03	94.56	95.5
GC1118 #3	98.93	79.04	79.9
GC1118 #4	99.74	94.59	94.8
GC1118 #5	99.75	91.67	91.9
GC1118 #6	99.68	94.79	95.1
GC1118 #7	99.90	91.71	91.8
GC1118 #8	99.50	93.89	94.4
GC1118 #9	99.81	79.16	79.3
GC1118 #10	98.61	94.92	96.3
GC1118 #11	99.03	79.76	80.5
GC1118 #12	99.66	94.26	94.6
GC1118 #13	99.76	80.38	80.6
GC1118 #14	99.59	94.70	95.1
GC1118 #15	99.82	61.96	62.1
GC1118 #16	99.65	94.42	94.8



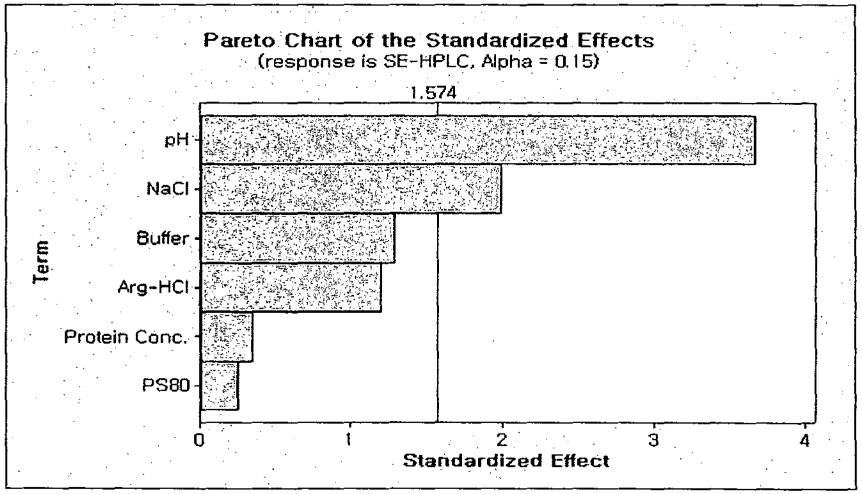
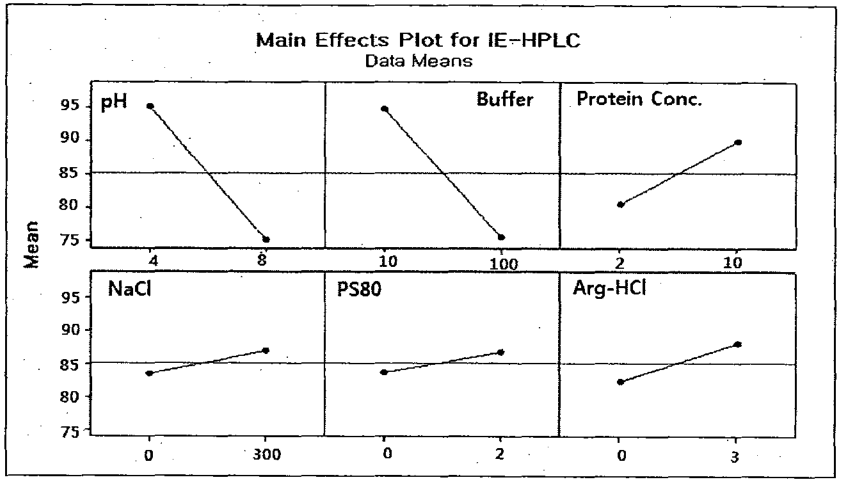


Fig. 3B

	Main Area% (Initial)	Main Area% (37°C/2W)	Main Area (compared to initial, %)
GC1118 #1	98.37	96.70	98.3
GC1118 #2	98.77	73.53	74.4
GC1118 #3	97.04	93.15	96.0
GC1118 #4	46.67	49.48	—
GC1118 #5	98.52	94.03	95.4
GC1118 #6	98.80	98.86	100.1
GC1118 #7	98.50	93.83	95.3
GC1118 #8	98.66	60.54	61.4
GC1118 #9	98.38	92.63	94.2
GC1118 #10	98.43	98.92	100.5
GC1118 #11	98.49	91.93	93.3
GC1118 #12	40.09	70.13	—
GC1118 #13	98.39	93.55	95.1
GC1118 #14	98.47	98.93	100.5
GC1118 #15	98.34	92.38	93.9
GC1118 #16	98.42	76.65	77.9



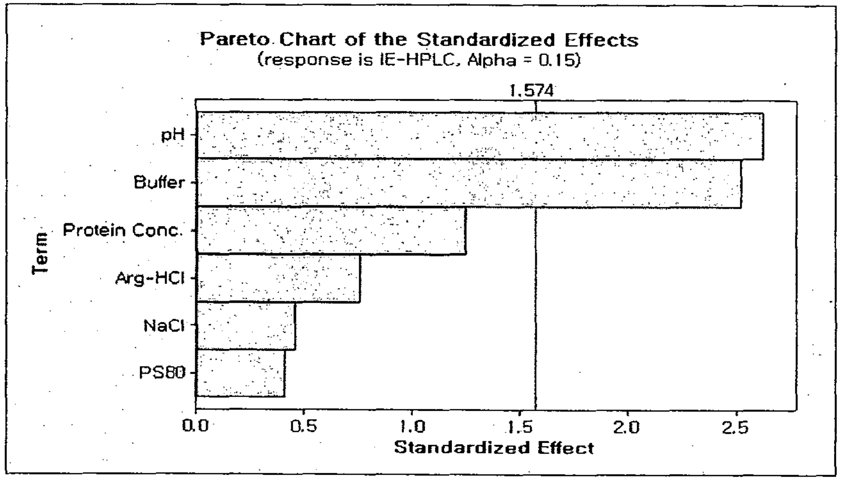


Fig. 4A

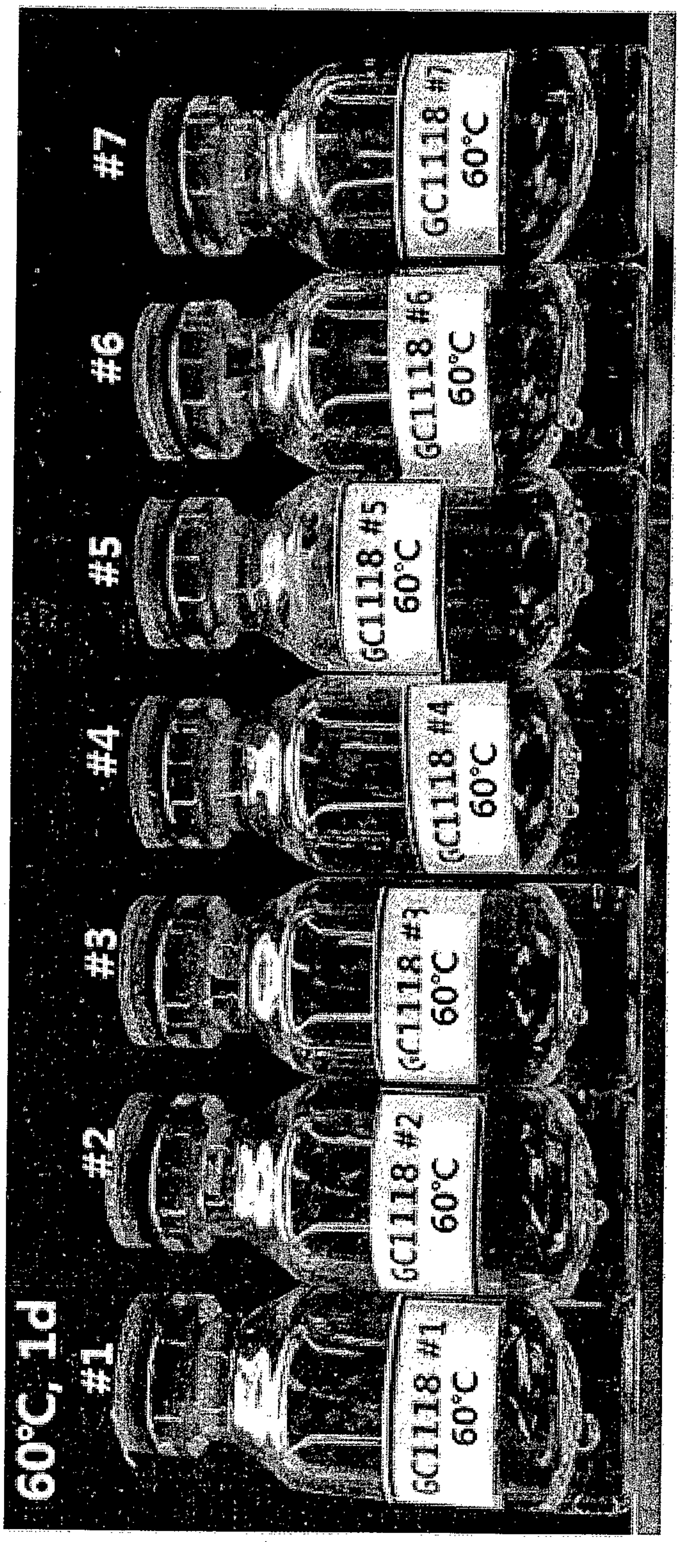

60°C, 1d
#1
#2
#3
#4
#5
#6
#7
GC1118 #1
60°C
GC1118 #2
60°C
GC1118 #3
60°C
GC1118 #4
60°C
GC1118 #5
60°C
GC1118 #6
60°C
GC1118 #7
60°C

Fig. 4B

Raw data

No	Turbidity Initial	Turbidity 60°C,1d	Turbidity (amount of change)
#1 w/o NaCl	0.034	0.064	0.0295
#2 110mM NaCl	0.035	0.066	0.0310
#3 3% Glycine	0.040	0.059	0.0185
#4 3% Arg-HCl	0.040	0.067	0.0270
#5 5% Mannitol	0.033	0.046	0.0135
#6 5% Sorbitol	0.036	0.064	0.0275
#7 5% Trehalose	0.061	0.085	0.0240

Turbidity graph

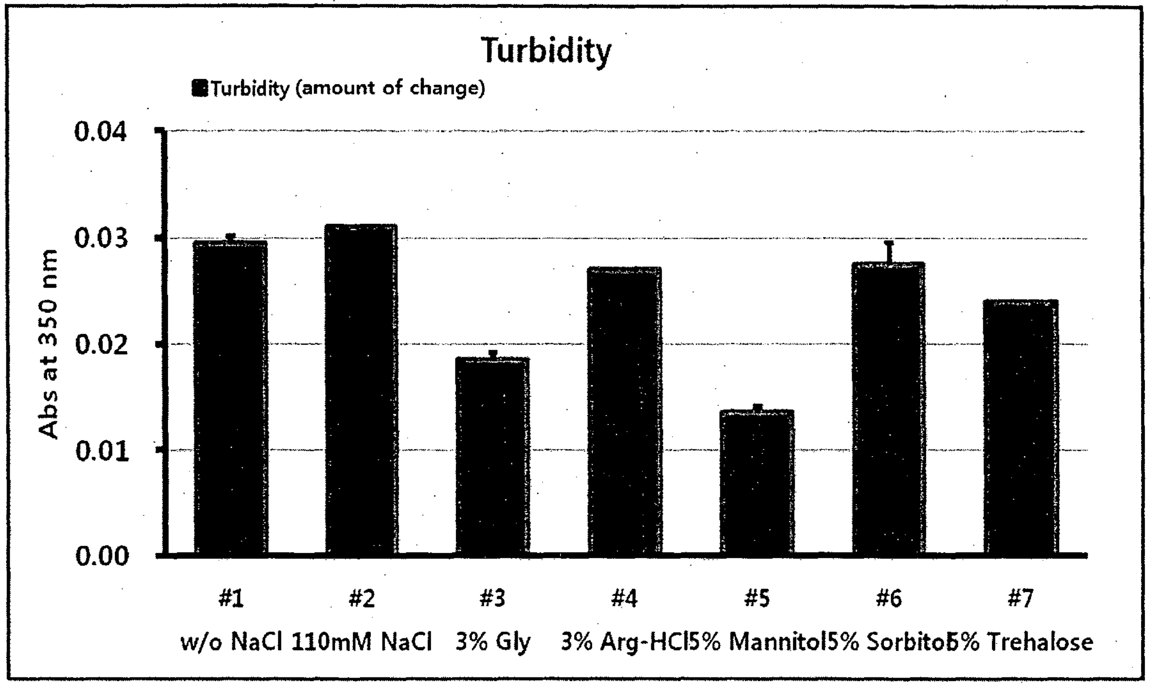


Fig. 4C

Raw data

No	Aggregates Initial (%)	Aggregates 60°C,1d (%)	Aggregates (amount of increase, %)
#1 w/o NaCl	0.0	3.4	3.4
#2 110mM NaCl	0.0	37.3	37.3
#3 3% Glycine	0.0	4.8	4.8
#4 3% Arg-HCl	0.0	47.2	47.2
#5 5% Mannitol	0.1	1.0	0.9
#6 5% Sorbitol	0.1	1.4	1.3
#7 5% Trehalose	2.1	25.6	23.5

DLS graph

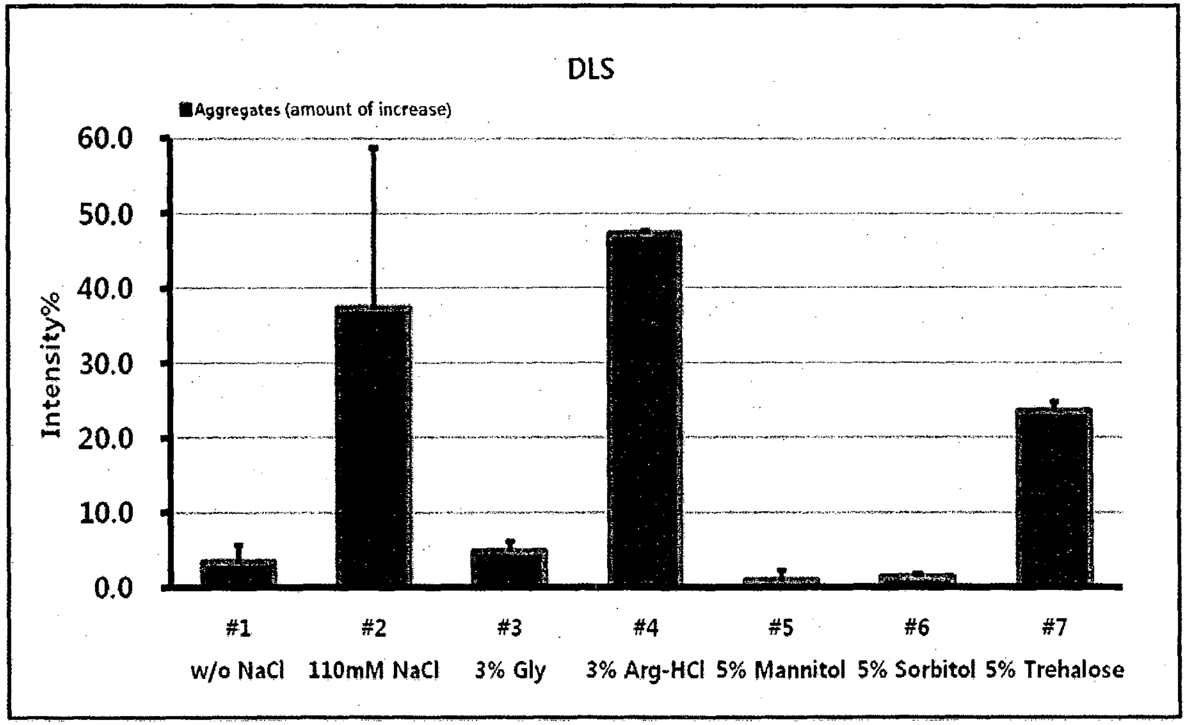


Fig. 5A

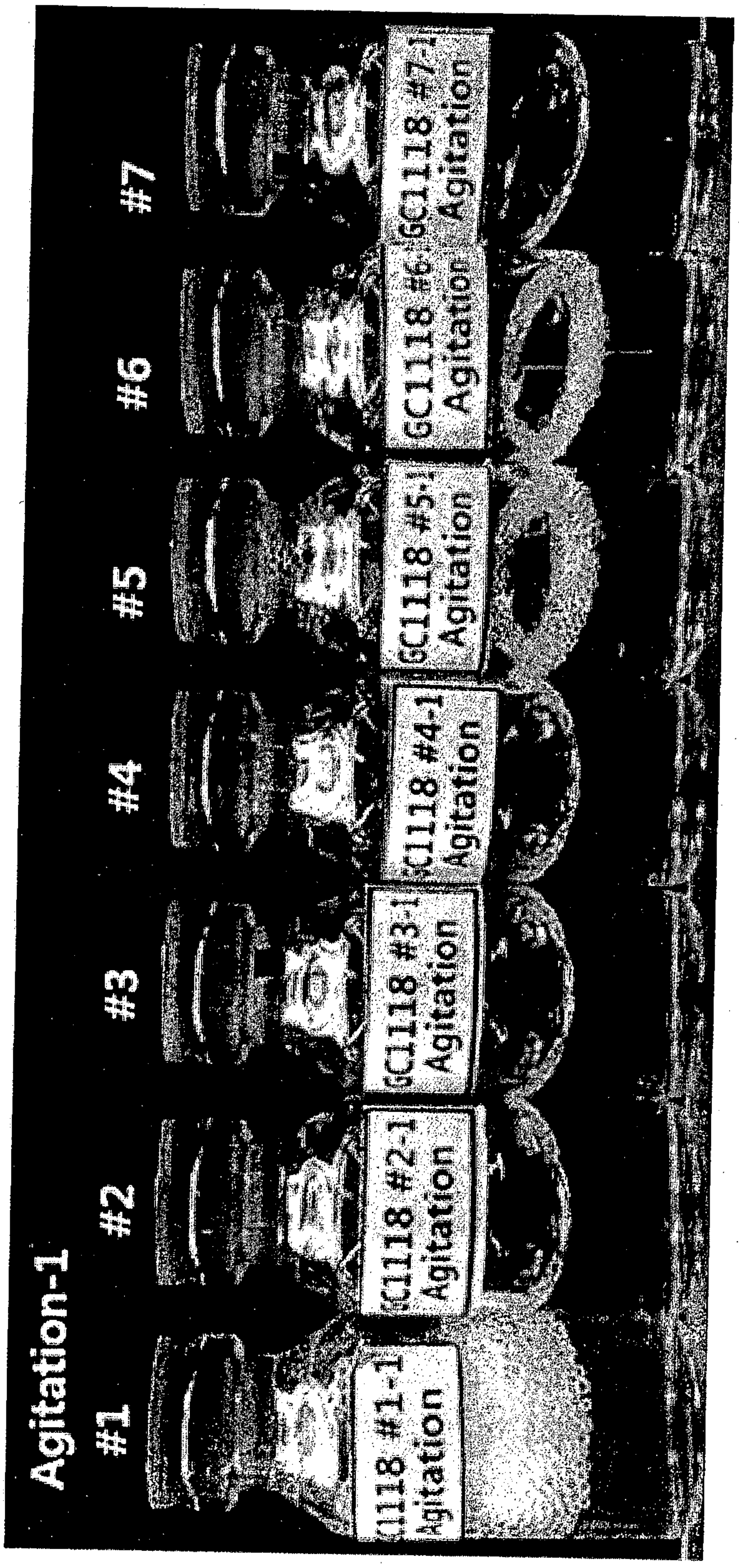


Fig. 5B

No	Turbidity (Abs 350nm)	
	Initial	Agitation
#1 w/o PS80	0.061	0.374
#2 0.05 mg/mL	0.063	0.069
#2 0.1 mg/mL	0.061	0.066
#3 0.2 mg/mL	0.064	0.064
#4 0.5 mg/mL	0.064	0.067
#5 1.0 mg/mL	0.065	0.069
#6 2.0 mg/mL	0.068	0.071

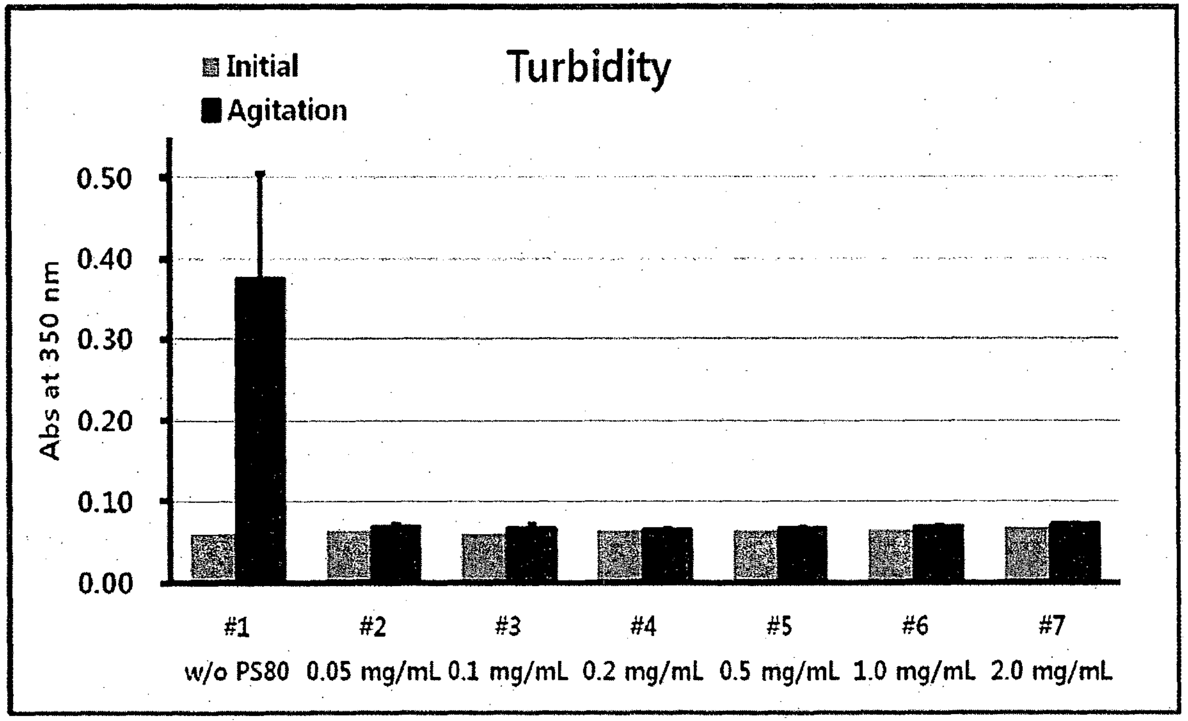
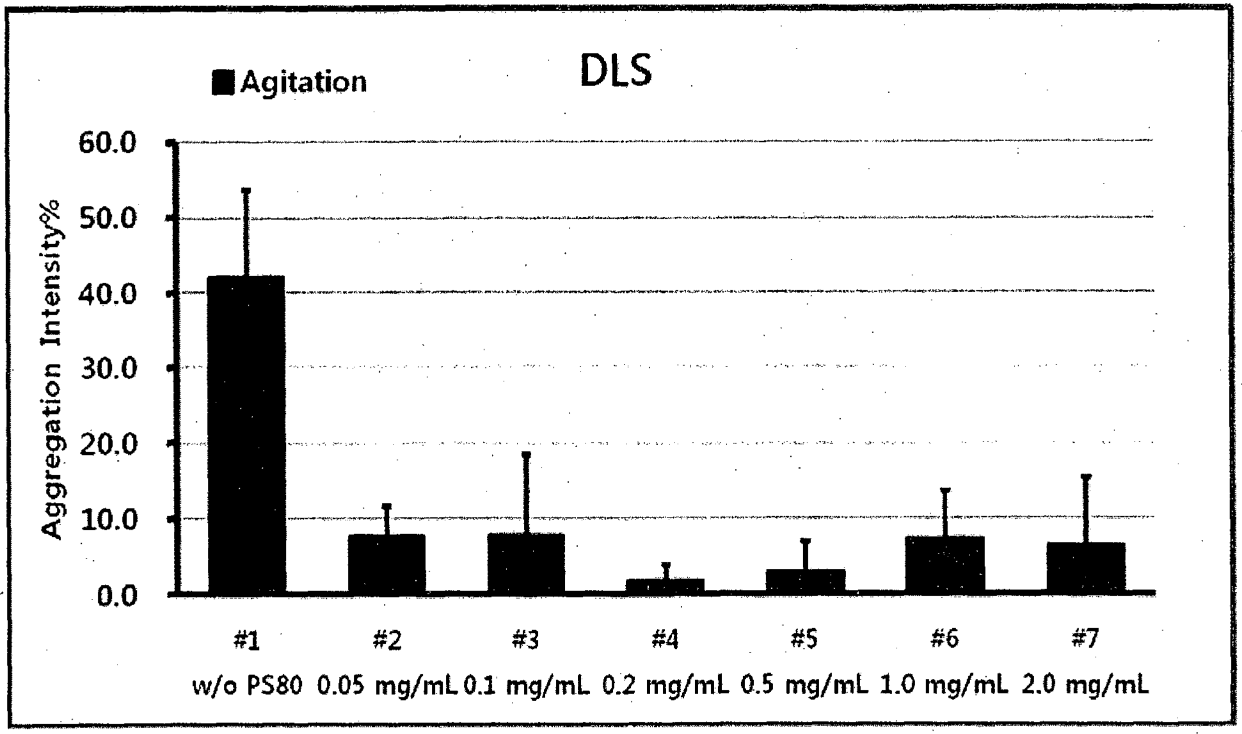


Fig. 5C



Form.	Initial (intensity%, > 11 nm)	Agitation (intensity%, > 11 nm)
#1 w/o PS80	0.0	42.0
#2 0.05 mg/mL	0.0	7.5
#3 0.1 mg/mL	0.0	7.65
#4 0.2 mg/mL	0.0	1.6
#5 0.5 mg/mL	0.0	2.9
#6 1.0 mg/mL	0.0	7.3
#7 2.0 mg/mL	0.0	6.35

Fig. 6A

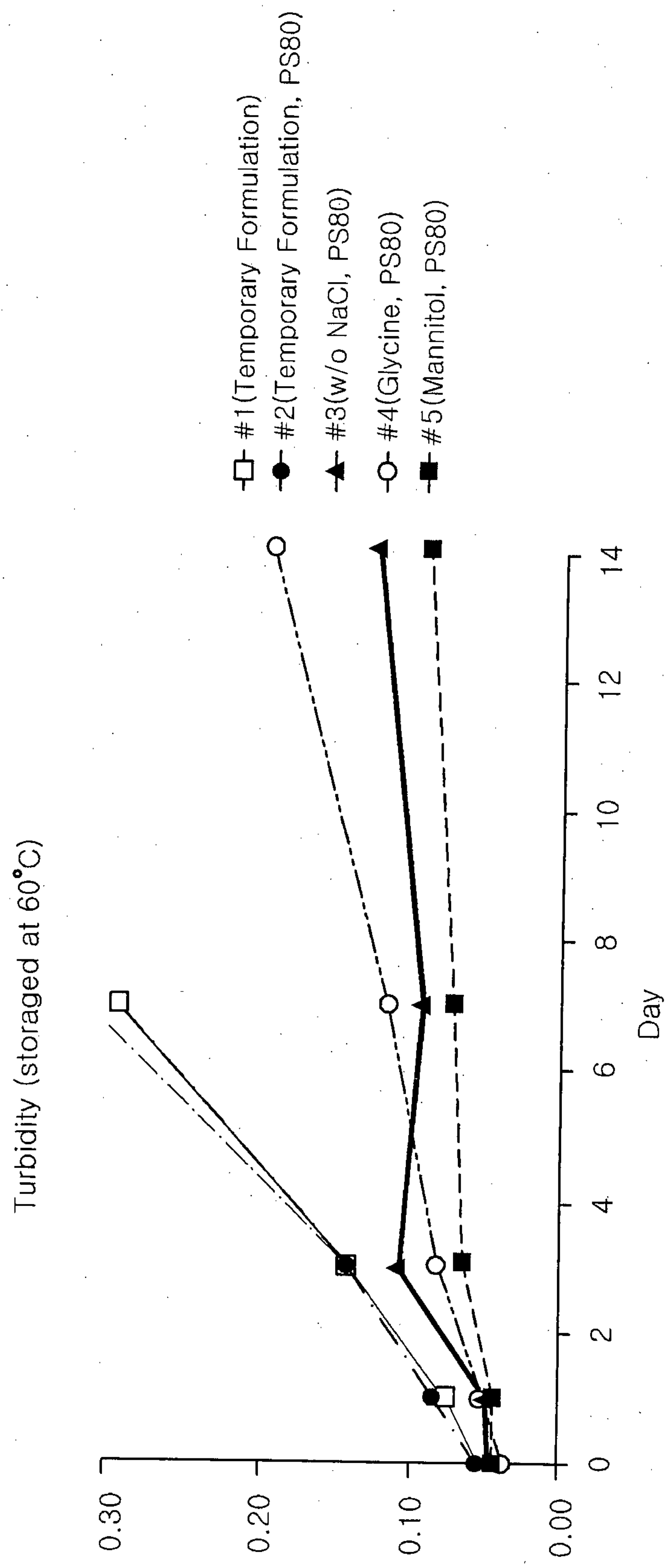

Turbidity (storaged at 60°C)
0.30
0.20
0.10
0.00
0
2
4
6
8
10
12
14
Day
#1(Temporary Formulation)
#2(Temporary Formulation, PS80)
#3(w/o NaCl, PS80)
#4(Glycine, PS80)
#5(Mannitol, PS80)

Fig. 6B

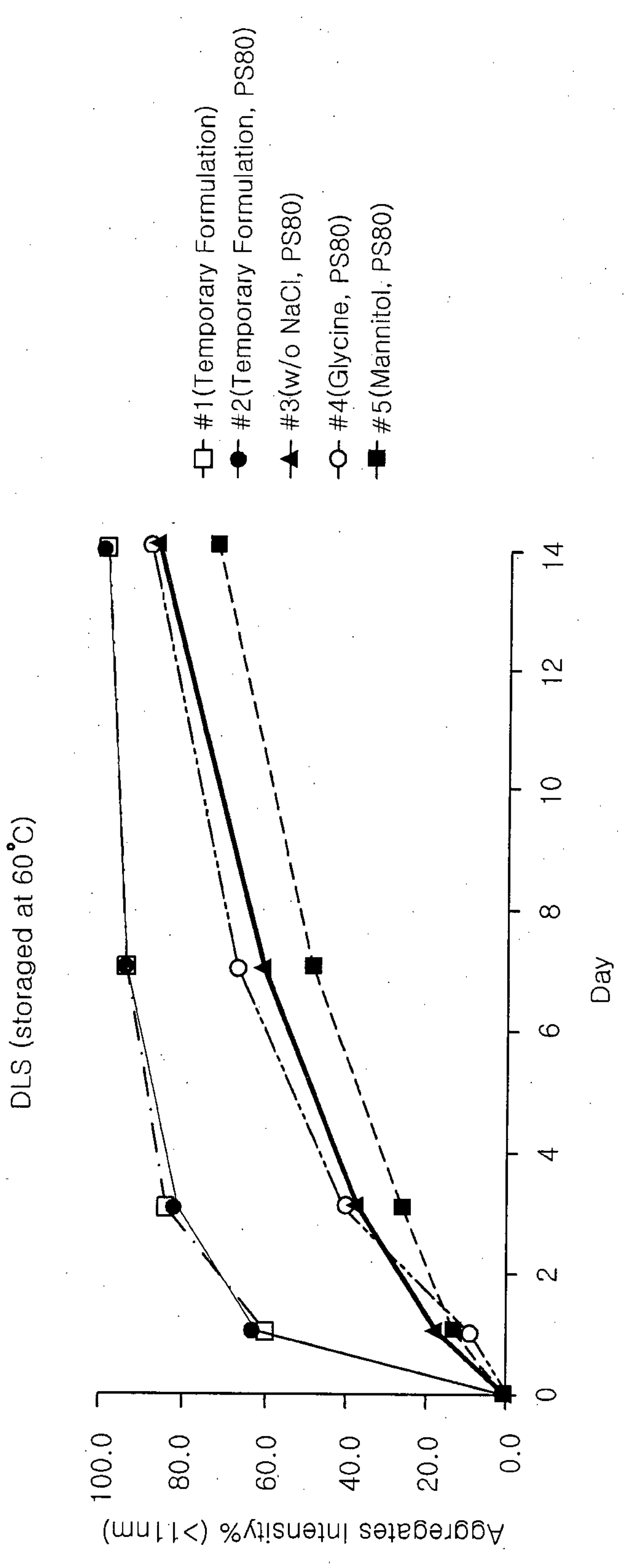

DLS (storaged at 60°C)
Aggregates Intensity% (>11nm)
100.0
80.0
60.0
40.0
20.0
0.0
0
2
4
6
8
10
12
14
Day
#1(Temporary Formulation)
#2(Temporary Formulation, PS80)
#3(w/o NaCl, PS80)
#4(Glycine, PS80)
#5(Mannitol, PS80)

Fig. 7A

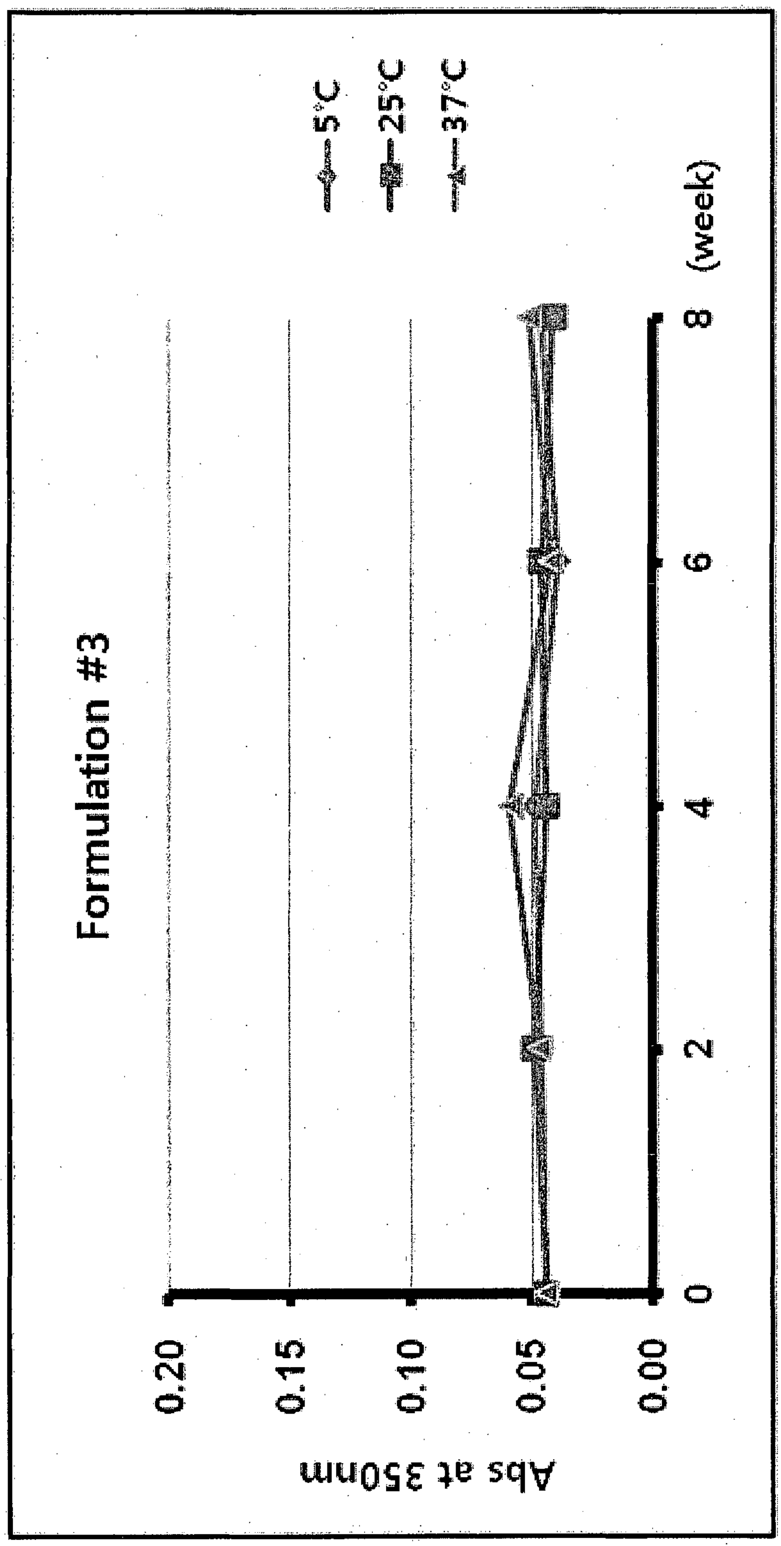

Formulation #3
Abs at 350nm
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
(week)
5°C
25°C
37°C

Fig. 7B

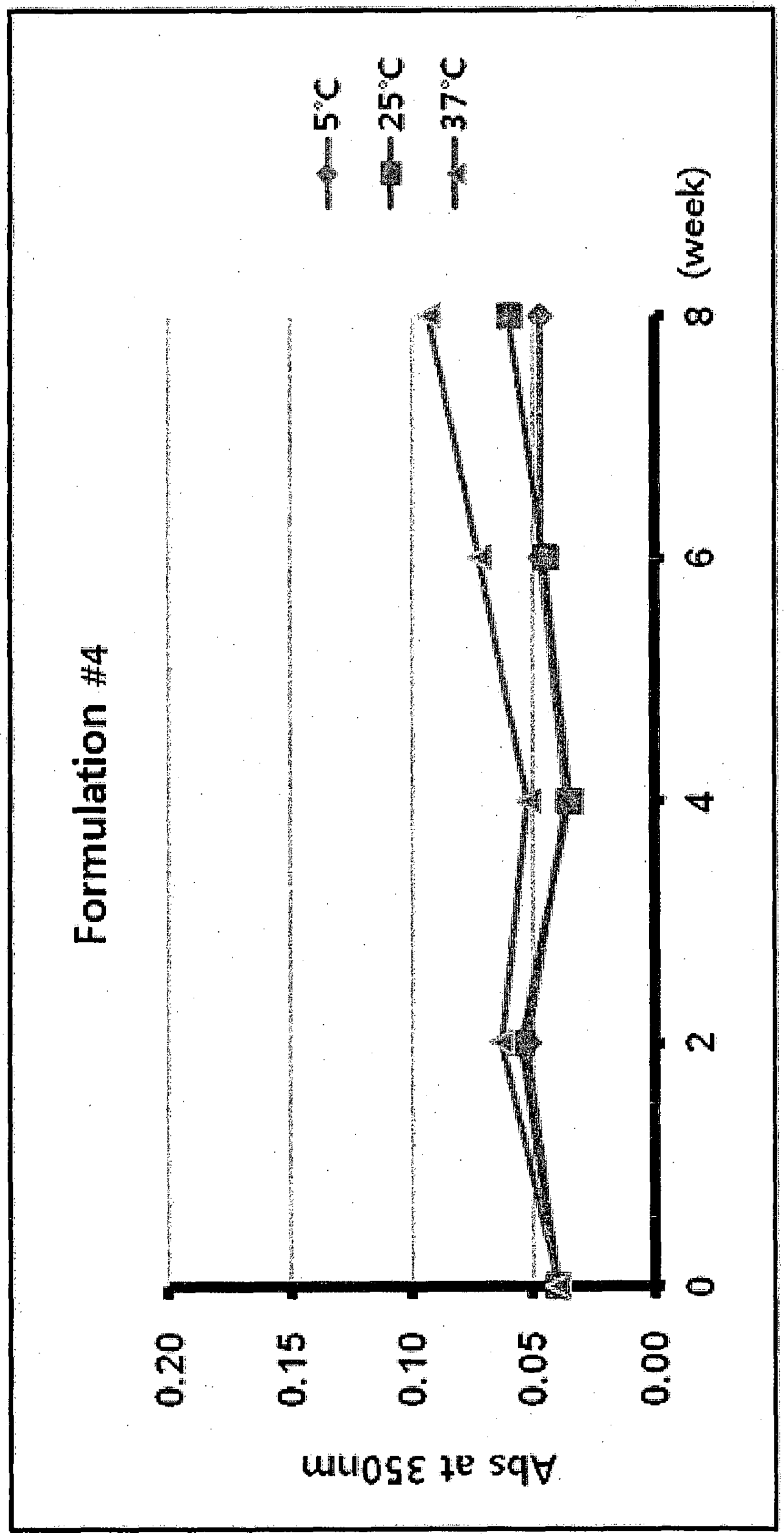
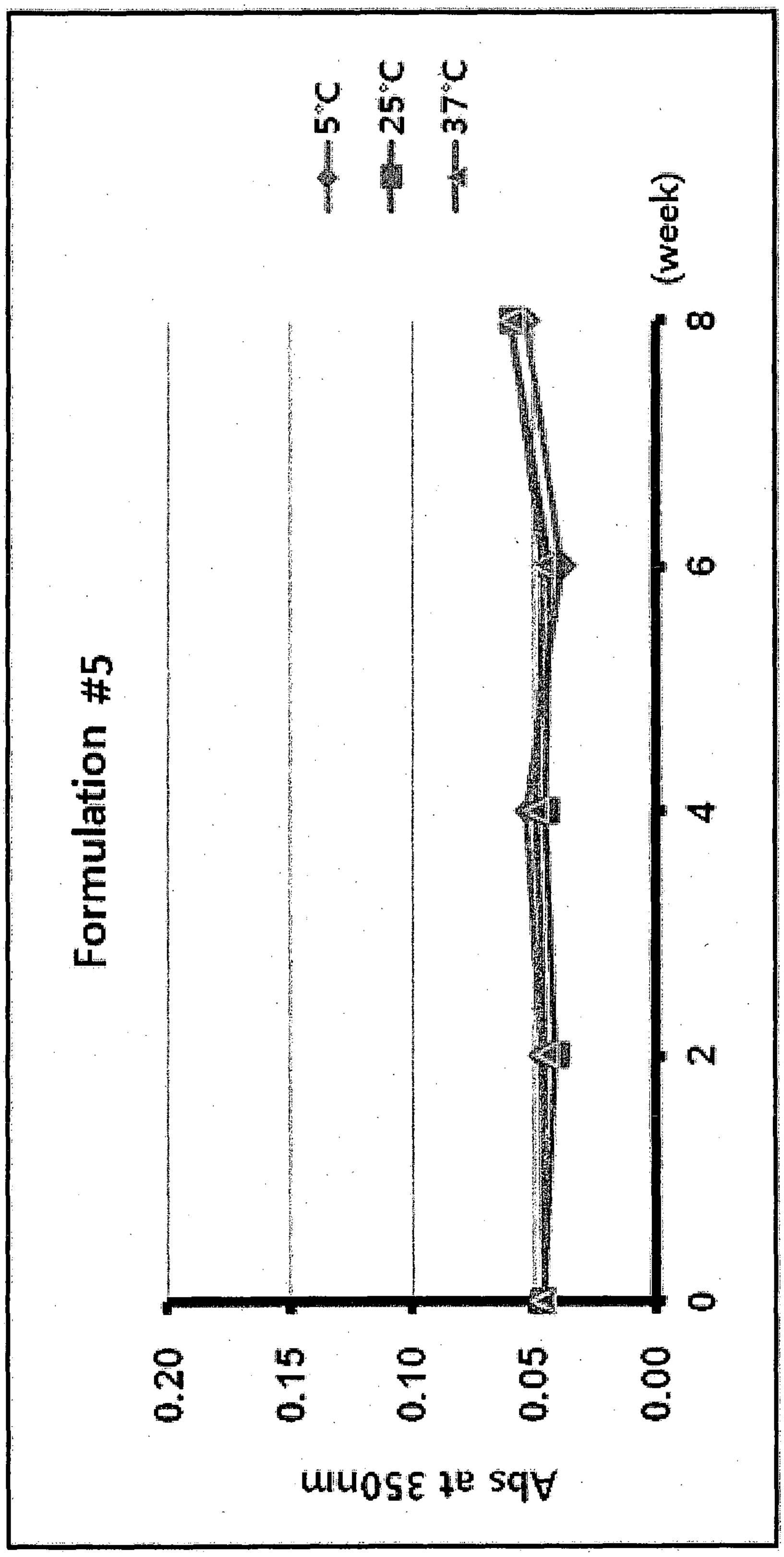

Formulation #4
Abs at 350nm
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
(week)
5°C
25°C
37°C

Fig. 7C


Formulation #5
Abs at 350nm
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
(week)
5°C
25°C
37°C

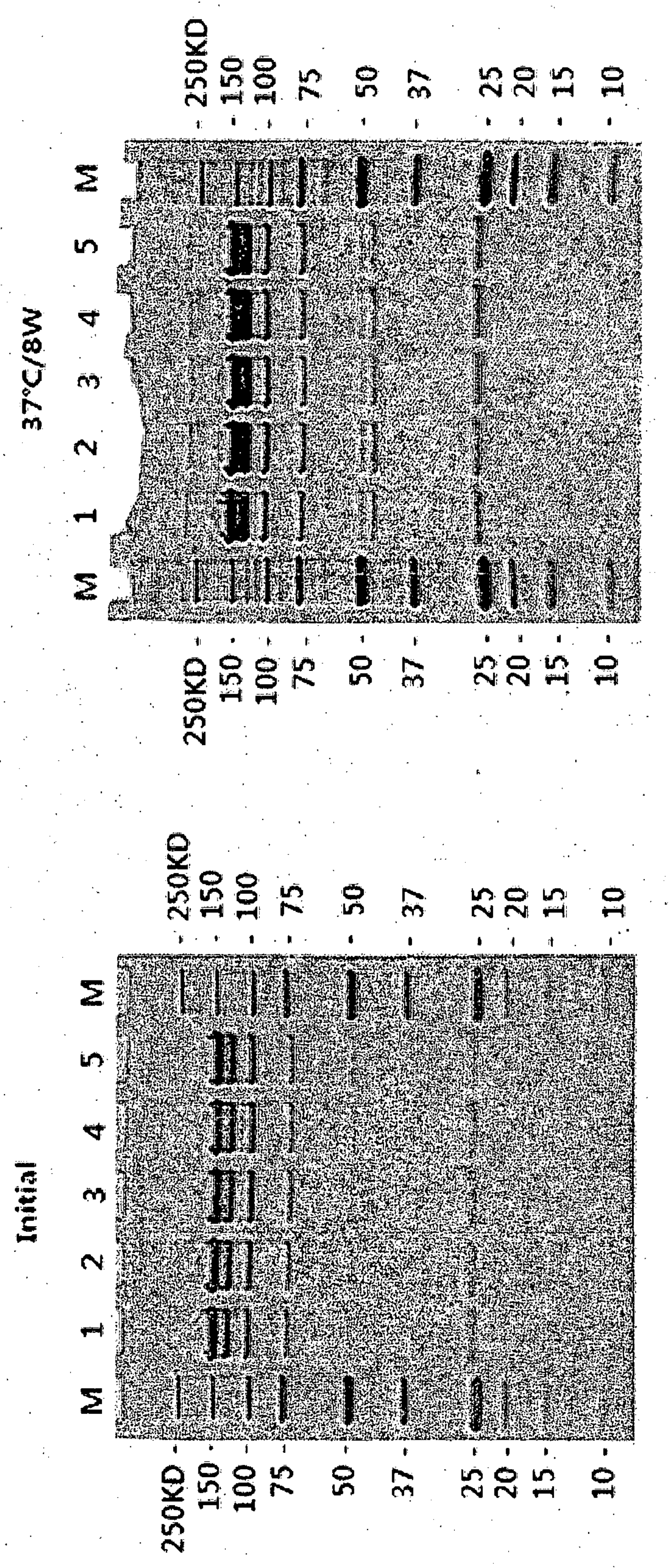

37°C/8W
M 1 2 3 4 5 M
250KD
150
100
75
50
37
25
20
15
10
Initial
M 1 2 3 4 5 M
250KD
150
100
75
50
37
25
20
15
10

Fig. 8A

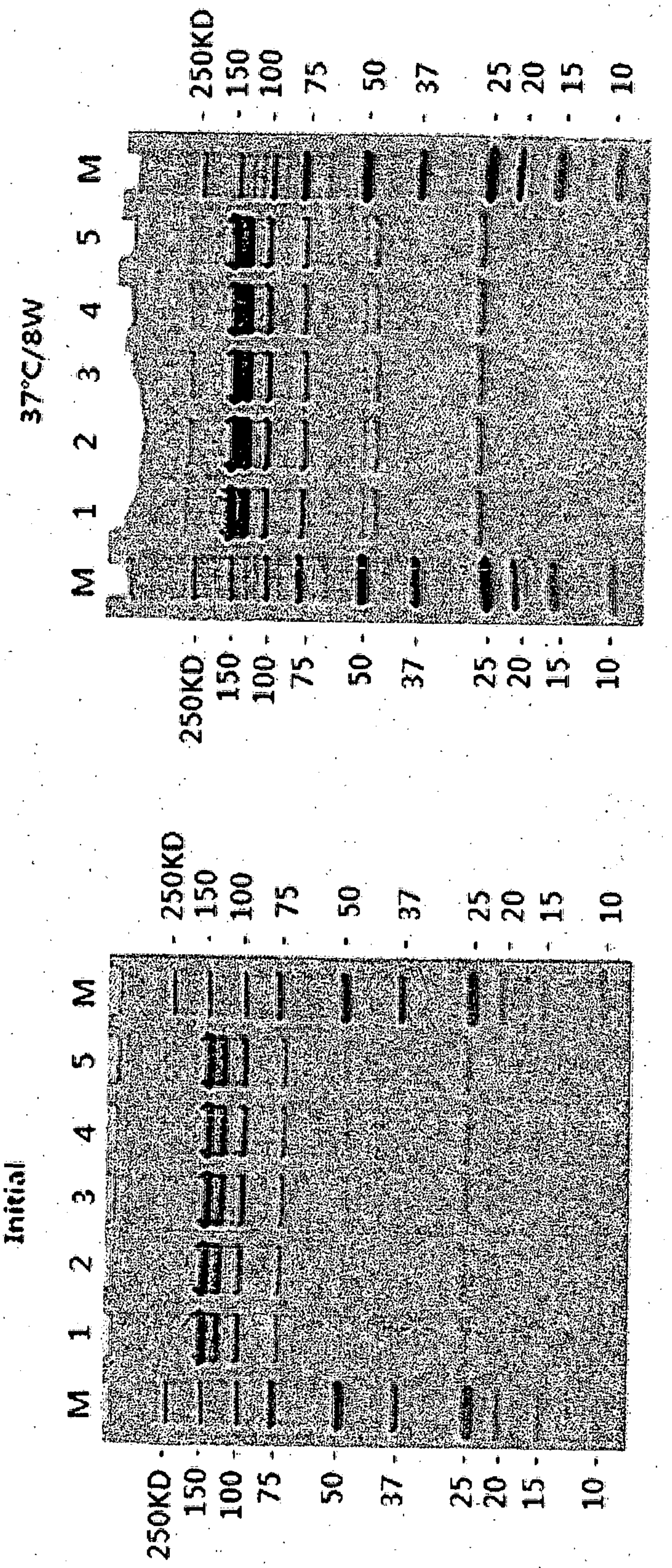

37°C/8W
M 1 2 3 4 5 M
250KD
150
100
75
50
37
25
20
15
10
Initial
M 1 2 3 4 5 M
250KD
150
100
75
50
37
25
20
15
10

Fig. 8B

Fig. 9

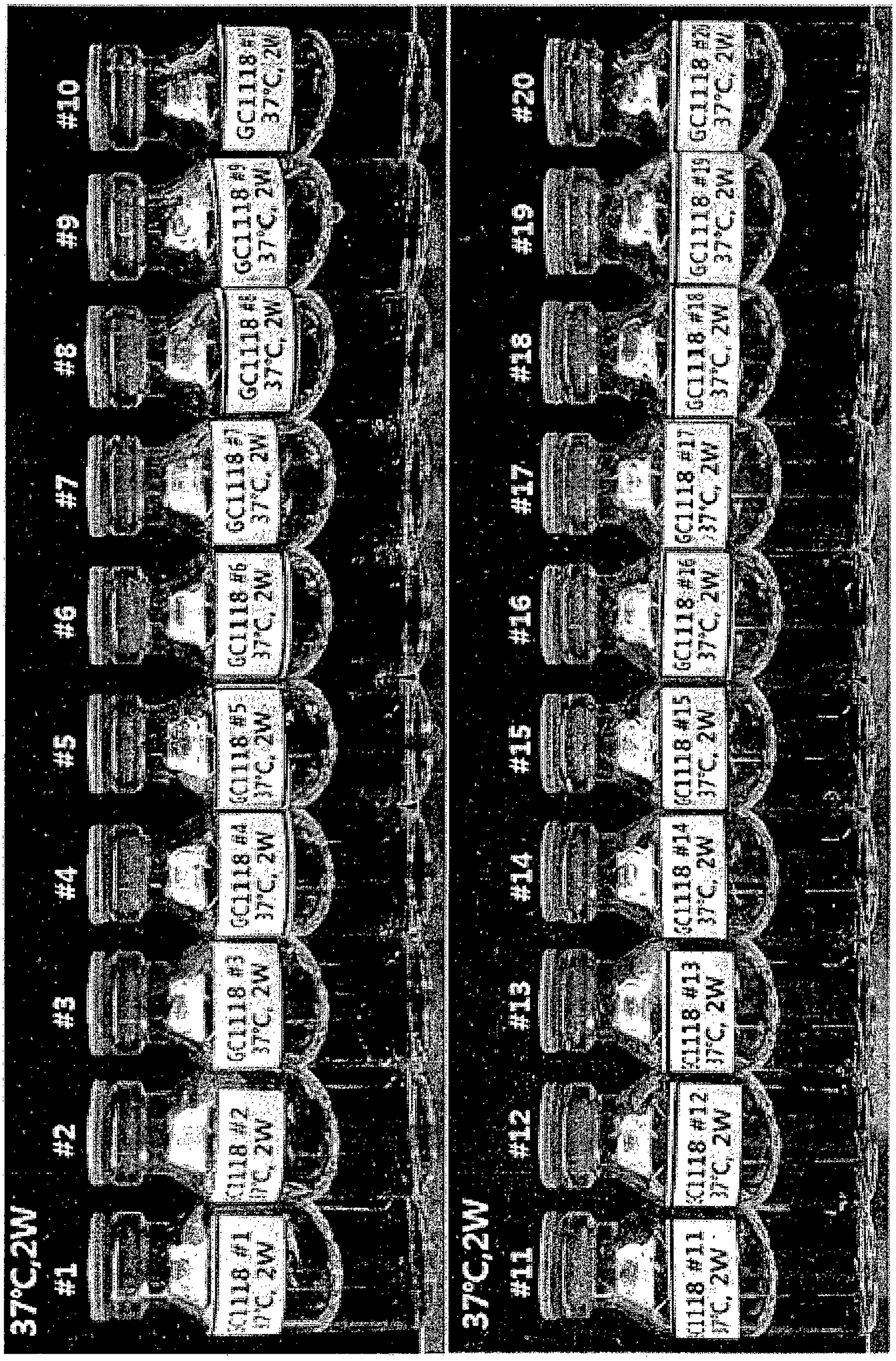


FIG. 10

Raw data

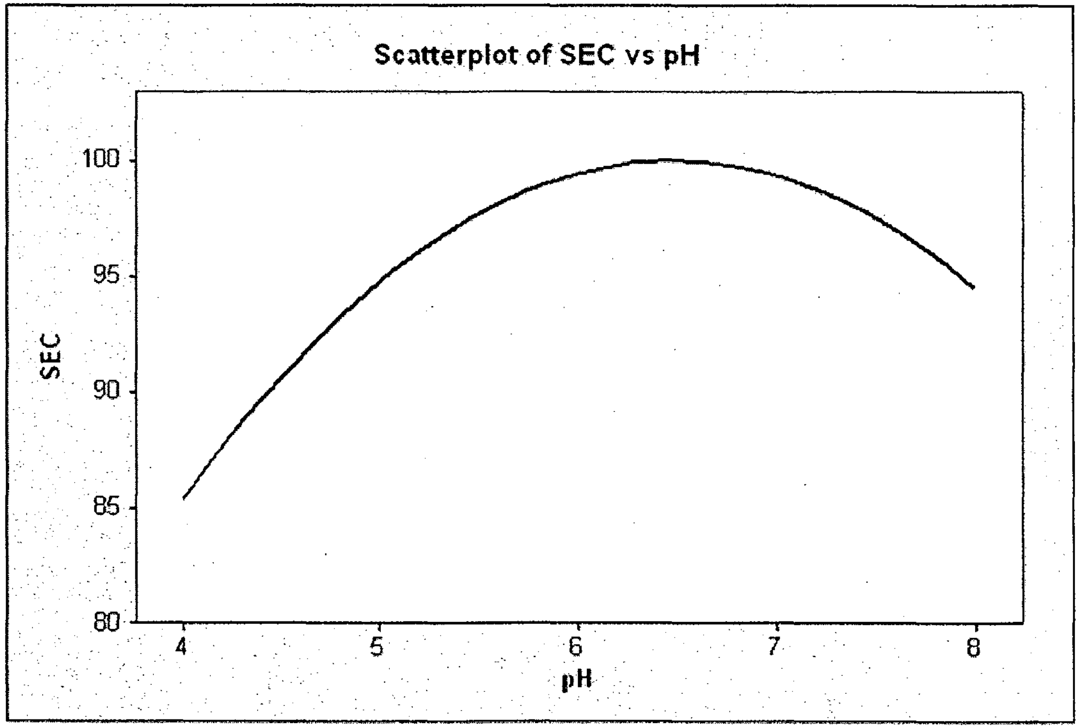
	Protein Concentration mg/mL (Initial)	Protein Concentration mg/mL (37°C/2W)	Protein Concentration (compared to Initial, %)
GC1118 #1	10.06	9.72	96.6
GC1118 #2	10.02	10.00	99.8
GC1118 #3	10.03	9.89	98.6
GC1118 #4	9.95	9.75	97.9
GC1118 #5	9.94	9.87	99.4
GC1118 #6	9.97	9.84	98.7
GC1118 #7	9.91	9.69	97.8
GC1118 #8	9.75	9.84	101.0
GC1118 #9	10.13	9.91	97.8
GC1118 #10	9.91	9.62	97.1
GC1118 #11	9.98	9.87	98.9
GC1118 #12	9.97	9.81	98.4
GC1118 #13	9.95	9.87	99.2
GC1118 #14	9.97	9.80	98.3
GC1118 #15	9.80	9.89	101.0
GC1118 #16	9.80	10.00	102.1
GC1118 #17	9.95	9.80	98.4
GC1118 #18	9.94	9.86	99.2
GC1118 #19	9.94	9.80	98.6
GC1118 #20	9.97	9.84	98.7

Fig. 11

One Factor Plot (using Minitab)

Raw data

	Main Area% (Initial)	Main Area% (37°C/2W)	Main % (compared to Initial)
GC1118 #1	99.64	97.76	98.1
GC1118 #2	99.48	97.83	98.3
GC1118 #3	99.70	97.67	98.0
GC1118 #4	99.43	97.68	98.2
GC1118 #5	99.66	97.15	97.5
GC1118 #6	99.55	98.03	98.5
GC1118 #7	99.63	97.33	97.7
GC1118 #8	99.55	97.85	98.3
GC1118 #9	99.66	76.31	76.6
GC1118 #10	99.47	97.09	97.6
GC1118 #11	99.57	97.99	98.4
GC1118 #12	99.60	98.00	98.4
GC1118 #13	99.58	98.06	98.5
GC1118 #14	99.62	97.96	98.3
GC1118 #15	99.60	98.05	98.4
GC1118 #16	99.57	98.01	98.4
GC1118 #17	99.63	98.00	98.4
GC1118 #18	99.59	98.03	98.4
GC1118 #19	99.59	98.08	98.5
GC1118 #20	99.59	98.07	98.5



- Model : Quadratic
- p-value : < 0.001
- R-Square : 0.6019

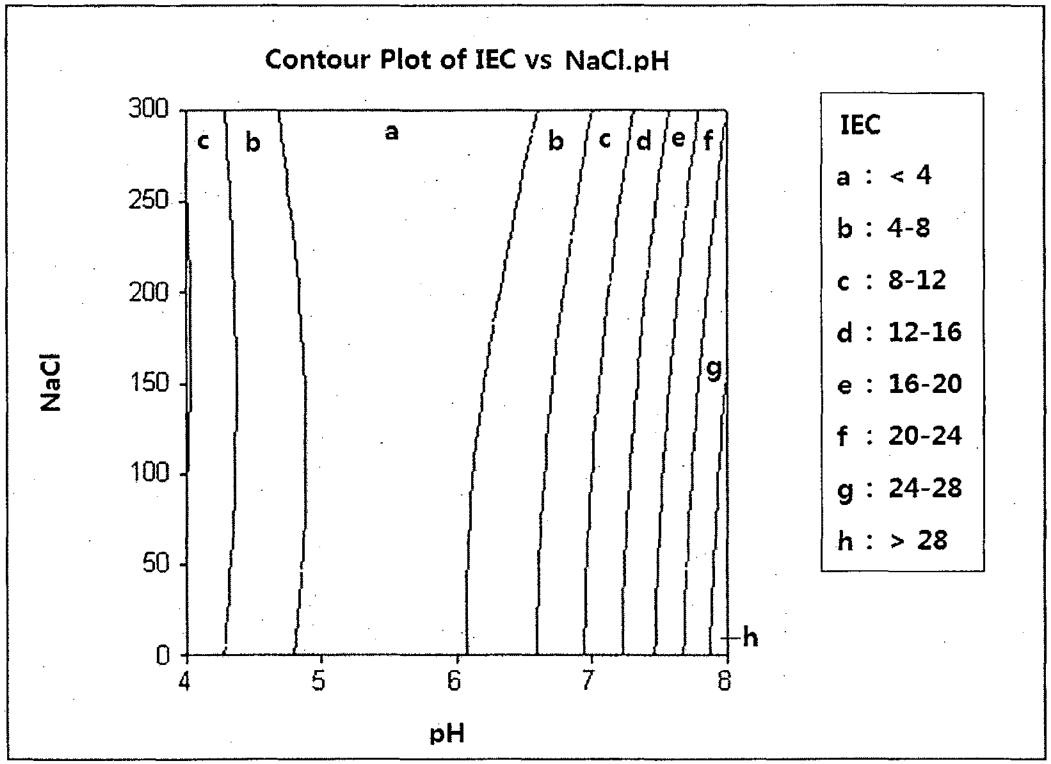
- Buffer : 55mM
- NaCl : 150mM

Fig. 12

Raw data

	Main Peak compared to Initial (amount of change)
GC1118 #1	5.32
GC1118 #2	12.73
GC1118 #3	5.30
GC1118 #4	14.19
GC1118 #5	3.51
GC1118 #6	9.24
GC1118 #7	4.25
GC1118 #8	9.67
GC1118 #9	10.40
GC1118 #10	31.94
GC1118 #11	2.14
GC1118 #12	3.02
GC1118 #13	3.48
GC1118 #14	2.64
GC1118 #15	2.39
GC1118 #16	2.06
GC1118 #17	3.77
GC1118 #18	4.27
GC1118 #19	4.41
GC1118 #20	3.33

Contour Plot (using Minitab)



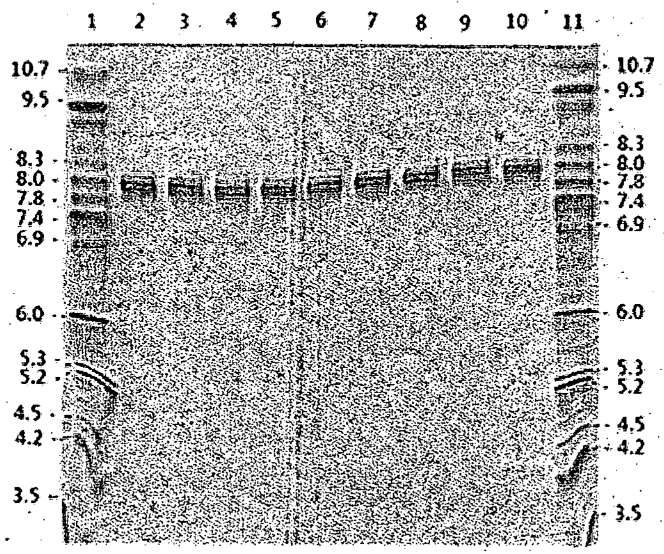
- Model : Quadratic
- p-value (P>F) : <0.0001
- R-Square : 0.9460
- Buffer : 55mM

Fig. 13A

Sample

Well #1	Marker
Well #2	Control
Well #3	GC1118 #1
Well #4	GC1118 #2
Well #5	GC1118 #3
Well #6	GC1118 #4
Well #7	GC1118 #5
Well #8	GC1118 #6
Well #9	GC1118 #7
Well #10	GC1118 #8
Well #11	Marker

Raw data (Initial)



Raw data (37°C, 2W)

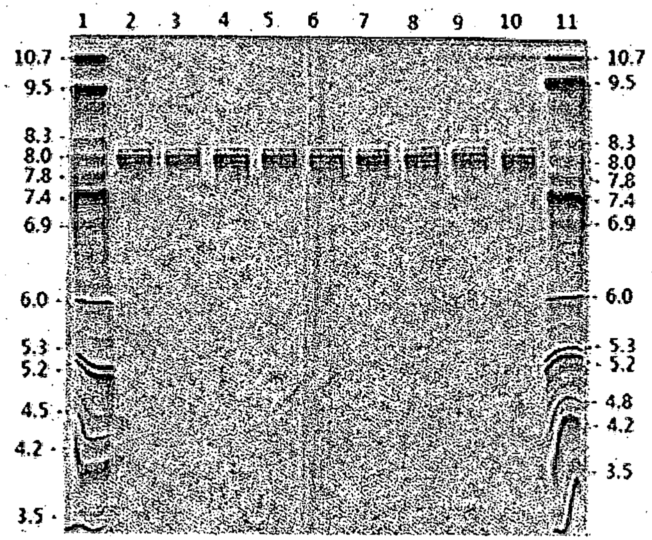


Fig. 13B

Sample

Well #1	Marker
Well #2	Control
Well #3	GC1118 #9
Well #4	GC1118 #10
Well #5	GC1118 #11
Well #6	GC1118 #12
Well #7	GC1118 #13
Well #8	GC1118 #14
Well #9	Control
Well #10	Marker

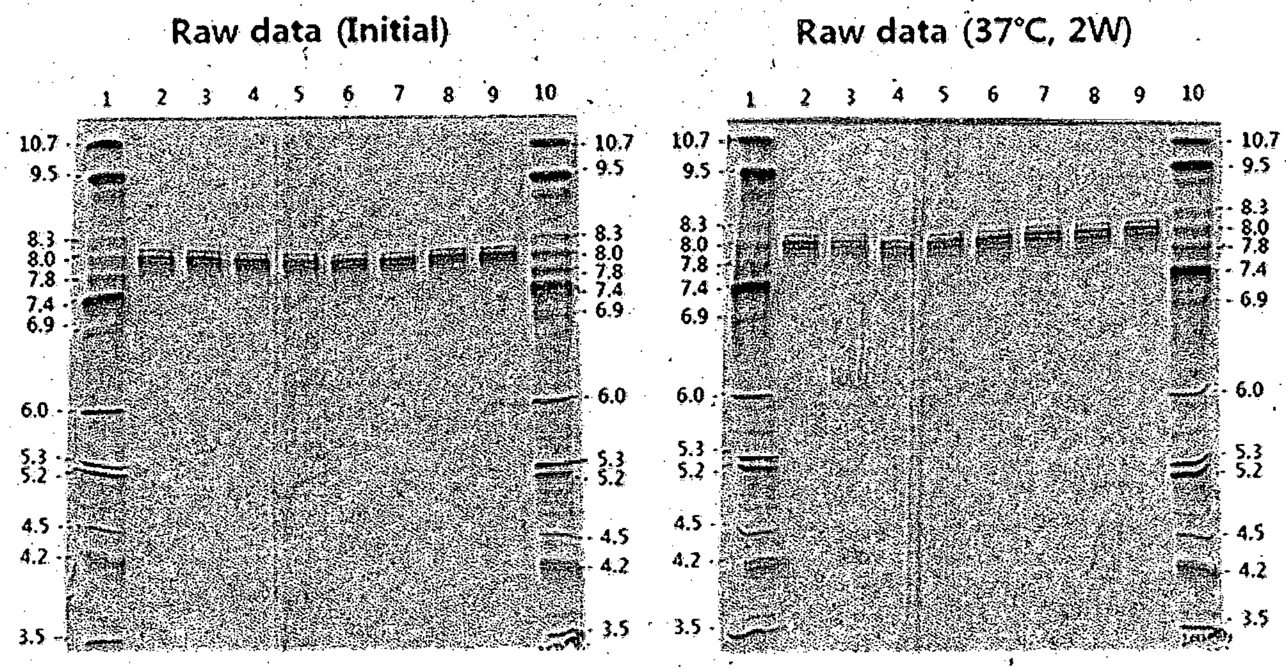
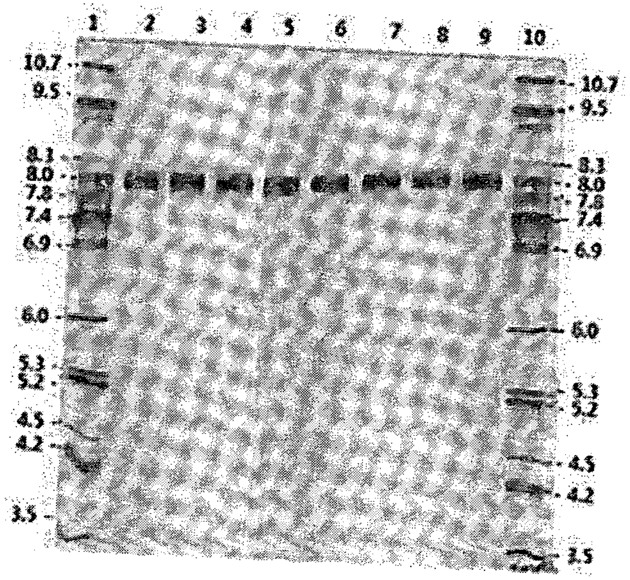


Fig. 13C

Sample

Well #1	Marker
Well #2	Control
Well #3	GC1118 #15
Well #4	GC1118 #16
Well #5	GC1118 #17
Well #6	GC1118 #18
Well #7	GC1118 #19
Well #8	GC1118 #20
Well #9	Control
Well #10	Marker

Raw data (Initial)



Raw data (37°C, 2W)

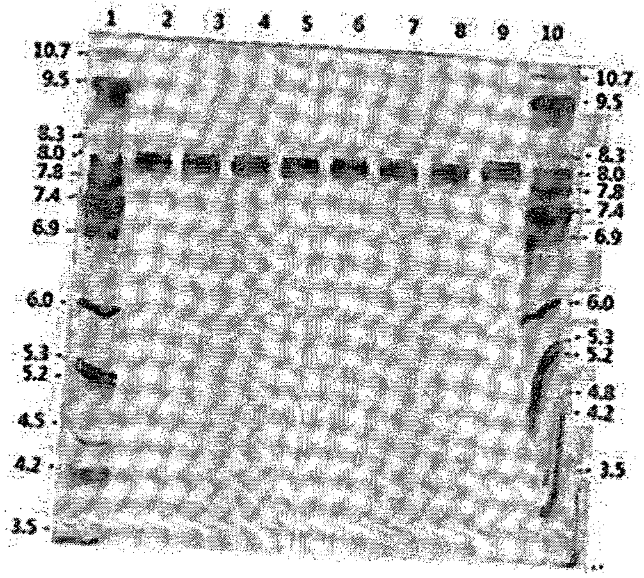
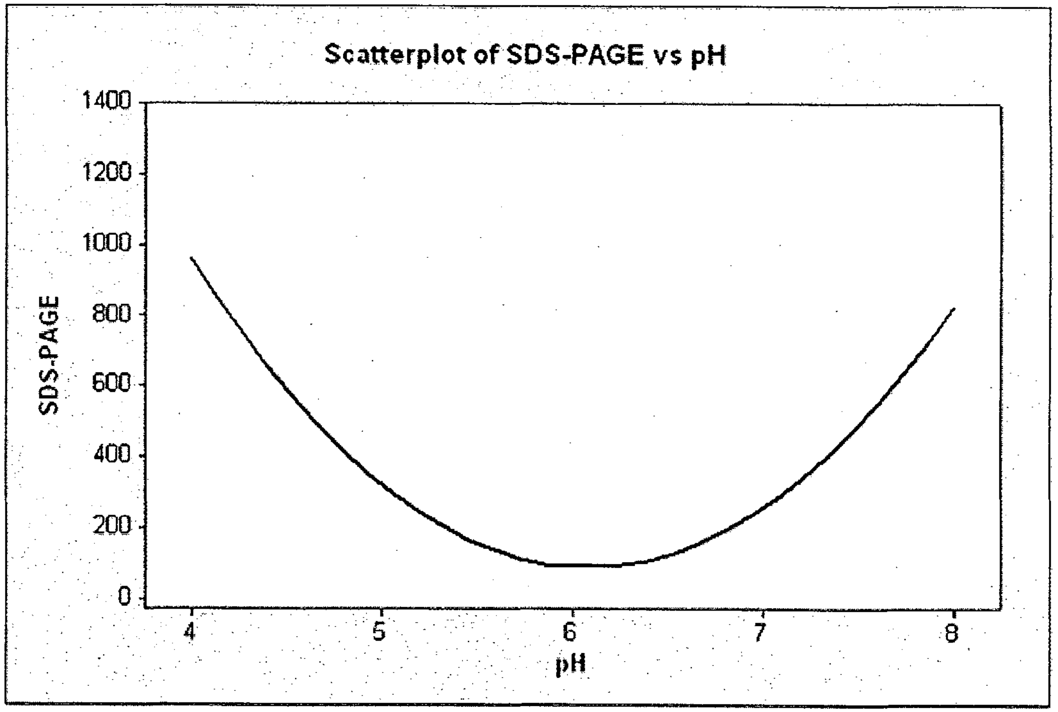


Fig. 14

Raw data

	Aggregates Intensity compared to Control, % (Non-Reducing, 37°C,2W)
GC1118 #1	111.4
GC1118 #2	329.1
GC1118 #3	164.4
GC1118 #4	526.8
GC1118 #5	171.1
GC1118 #6	312.5
GC1118 #7	141.6
GC1118 #8	348.3
GC1118 #9	1493.7
GC1118 #10	610.3
GC1118 #11	223.8
GC1118 #12	201.3
GC1118 #13	250.2
GC1118 #14	196.7
GC1118 #15	138.3
GC1118 #16	90.8
GC1118 #17	98.3
GC1118 #18	98.2
GC1118 #19	93.2
GC1118 #20	77.8

One Factor Plot (using Minitab)



- Model : Quadratic
- p-value : < 0.001
- R-Square : 0.6257
- Buffer : 55mM
- NaCl : 150mM

Fig. 15

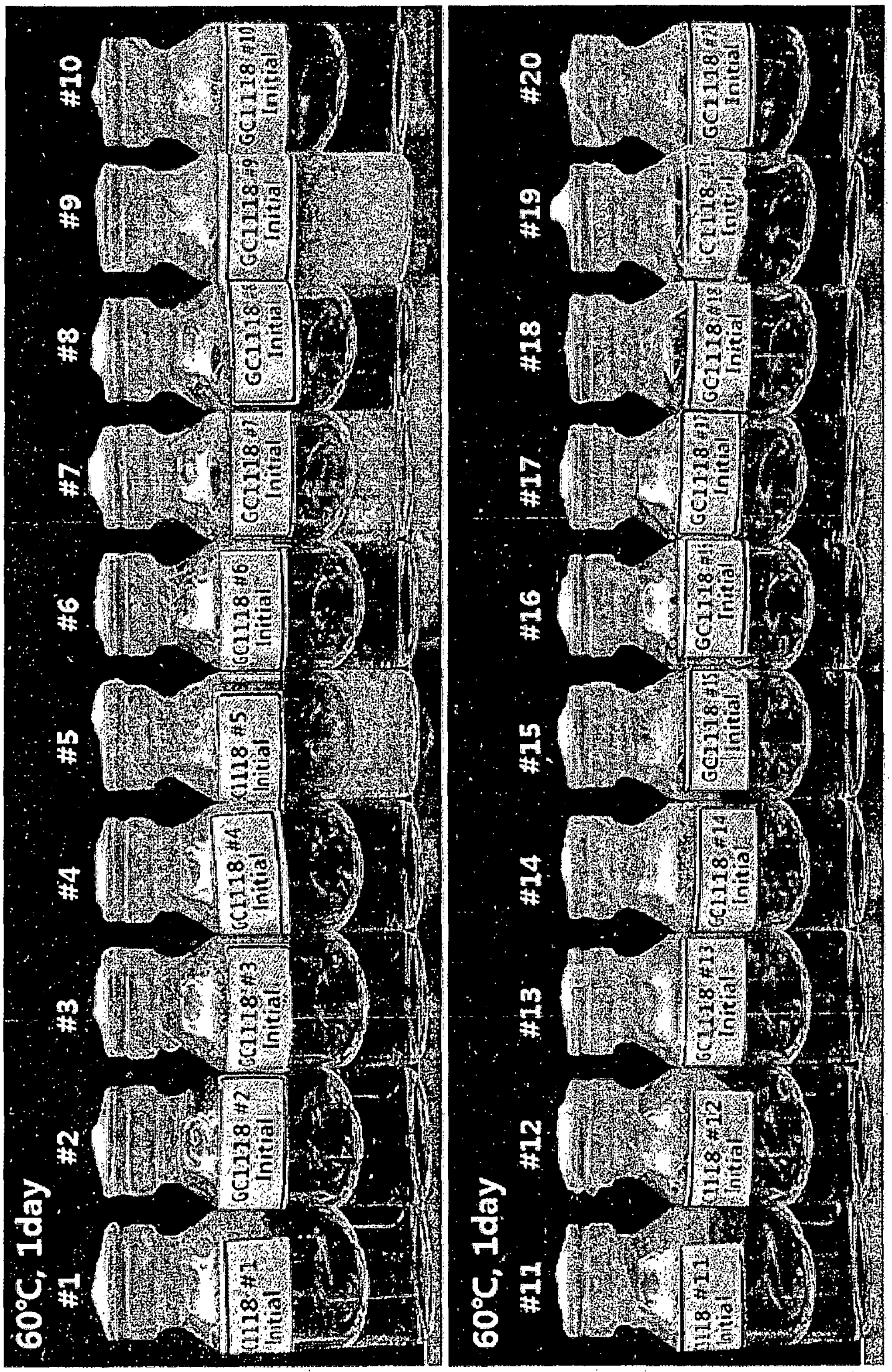
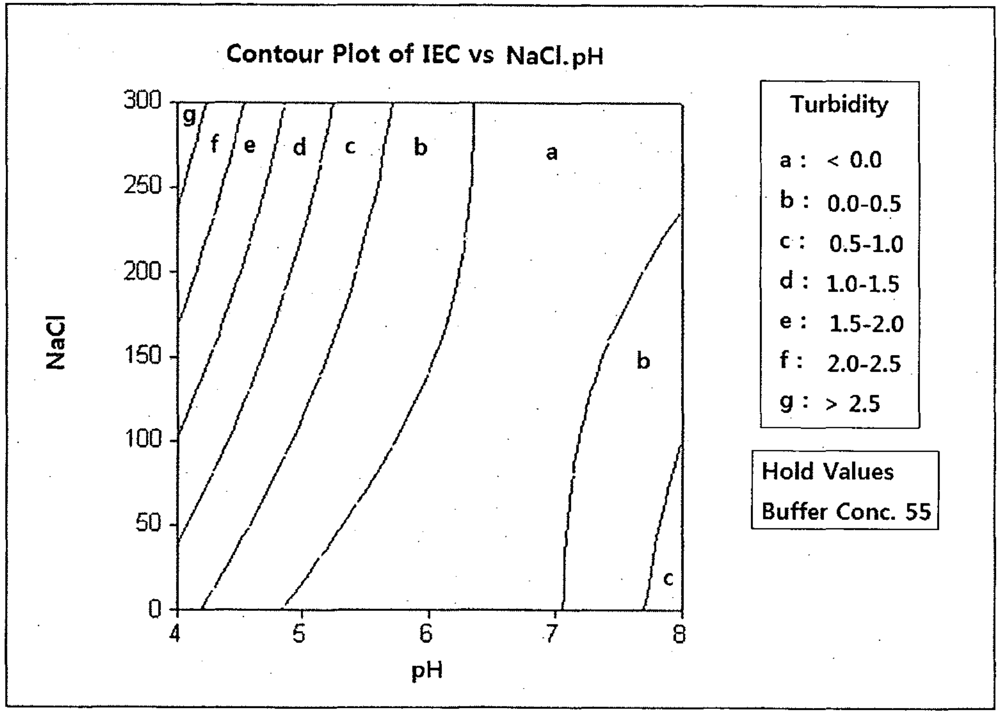

60°C, 1day
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
GC1118 #2 Initial
GC1118 #4 Initial
GC1118 #6 Initial
GC1118 #7 Initial
GC1118 #9 Initial
60°C, 1day
#11
#12
#13
#14
#15
#16
#17
#18
#19
#20

Fig. 16

Raw data

	Turbidity (Initial)	Turbidity (60℃/1d)	Turbidity (amount of change)
GC1118 #1	0.045	0.072	0.0270
GC1118 #2	0.046	0.058	0.0120
GC1118 #3	0.043	0.105	0.0620
GC1118 #4	0.045	0.057	0.0120
GC1118 #5	0.045	1.806	1.7605
GC1118 #6	0.043	0.061	0.0180
GC1118 #7	0.043	0.701	0.6575
GC1118 #8	0.044	0.060	0.0160
GC1118 #9	0.044	2.323	2.2785
GC1118 #10	0.048	0.064	0.0160
GC1118 #11	0.047	0.063	0.0160
GC1118 #12	0.046	0.062	0.0160
GC1118 #13	0.043	0.047	0.0040
GC1118 #14	0.044	0.063	0.0190
GC1118 #15	0.044	0.060	0.0160
GC1118 #16	0.044	0.061	0.0170
GC1118 #17	0.044	0.062	0.0180
GC1118 #18	0.045	0.064	0.0190
GC1118 #19	0.045	0.061	0.0160
GC1118 #20	0.044	0.062	0.0180

Contour Plot (using Minitab)



- Model : Quadratic
- p-value : 0.001
- R-Square : 0.8296

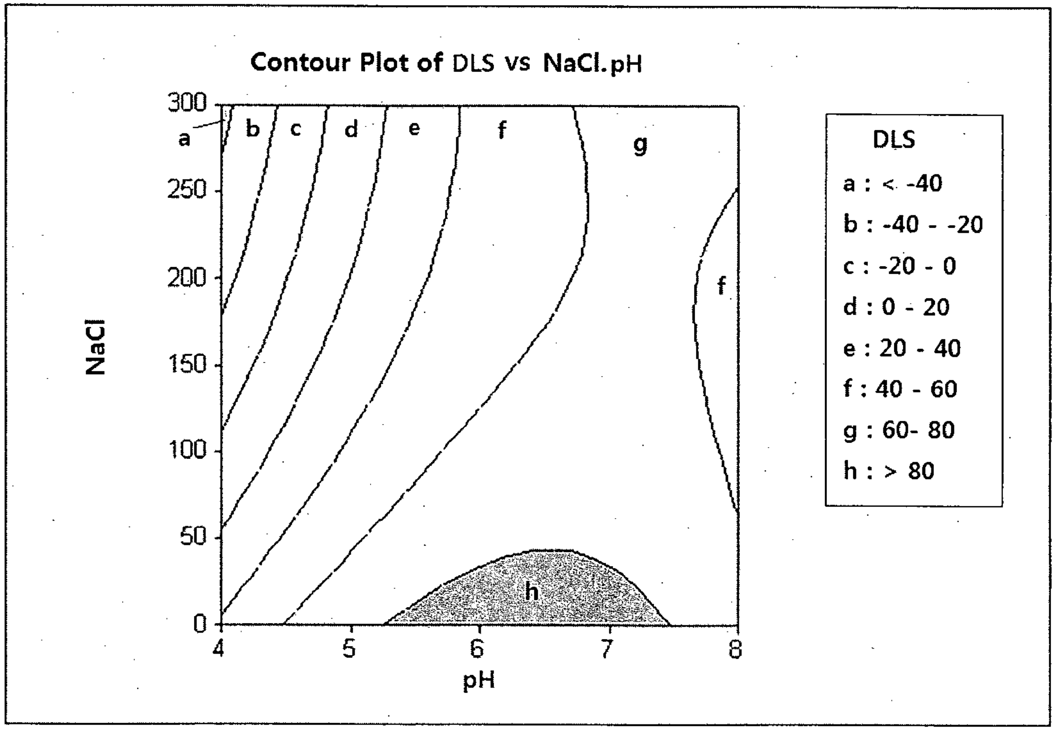
- Buffer : 55mM

Fig. 17

Raw data

	Monomer Intensity% (Initial)	Monomer Intensity% (60°C/1d)	Intensity (compared to Initial, %)
GC1118 #1	100.0	53.4	53.4
GC1118 #2	100.0	71.3	71.3
GC1118 #3	100.0	28.7	28.7
GC1118 #4	100.0	71.1	71.1
GC1118 #5	100.0	0.0	0.0
GC1118 #6	100.0	58.4	58.4
GC1118 #7	100.0	0.0	0.0
GC1118 #8	100.0	62.2	62.2
GC1118 #9	100.0	0.0	0.0
GC1118 #10	100.0	56.3	56.3
GC1118 #11	100.0	61.2	61.2
GC1118 #12	100.0	59.1	59.1
GC1118 #13	100.0	96.8	96.8
GC1118 #14	100.0	50.1	50.1
GC1118 #15	100.0	61.1	61.1
GC1118 #16	100.0	55.9	55.9
GC1118 #17	100.0	53.8	53.8
GC1118 #18	100.0	55.0	55.0
GC1118 #19	100.0	54.4	54.4
GC1118 #20	100.0	58.2	58.2

Contour Plot (using Minitab)



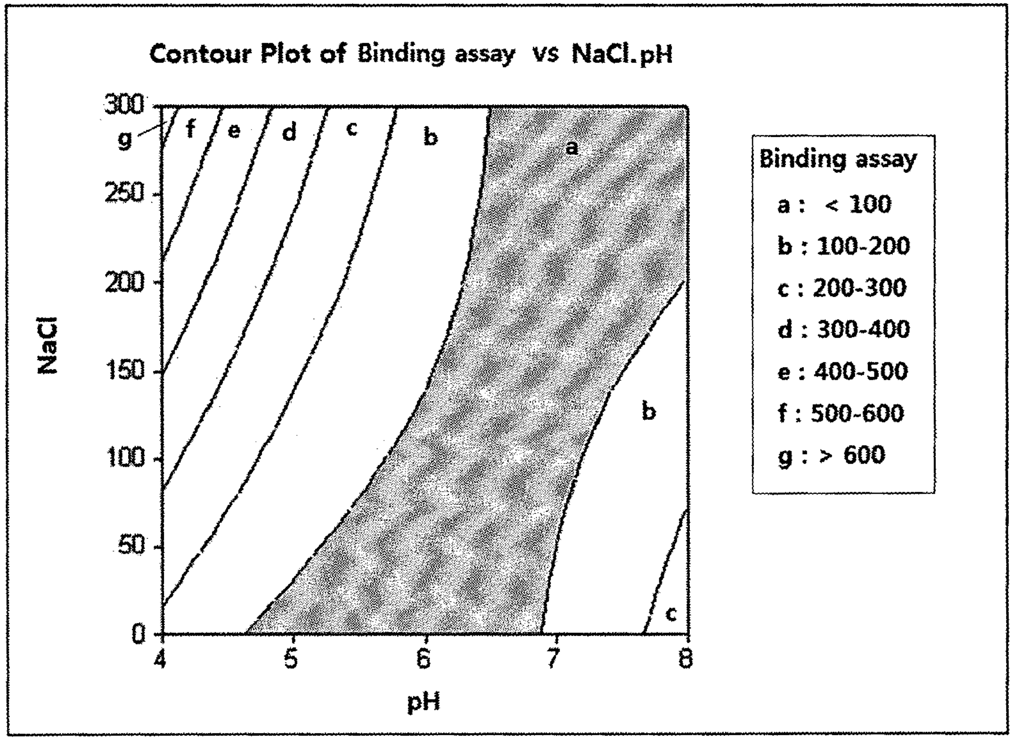
- Model : Quadratic
- p-value : <0.001
- R-Square : 0.9286
- Buffer : 55mM

Fig. 18

Raw data

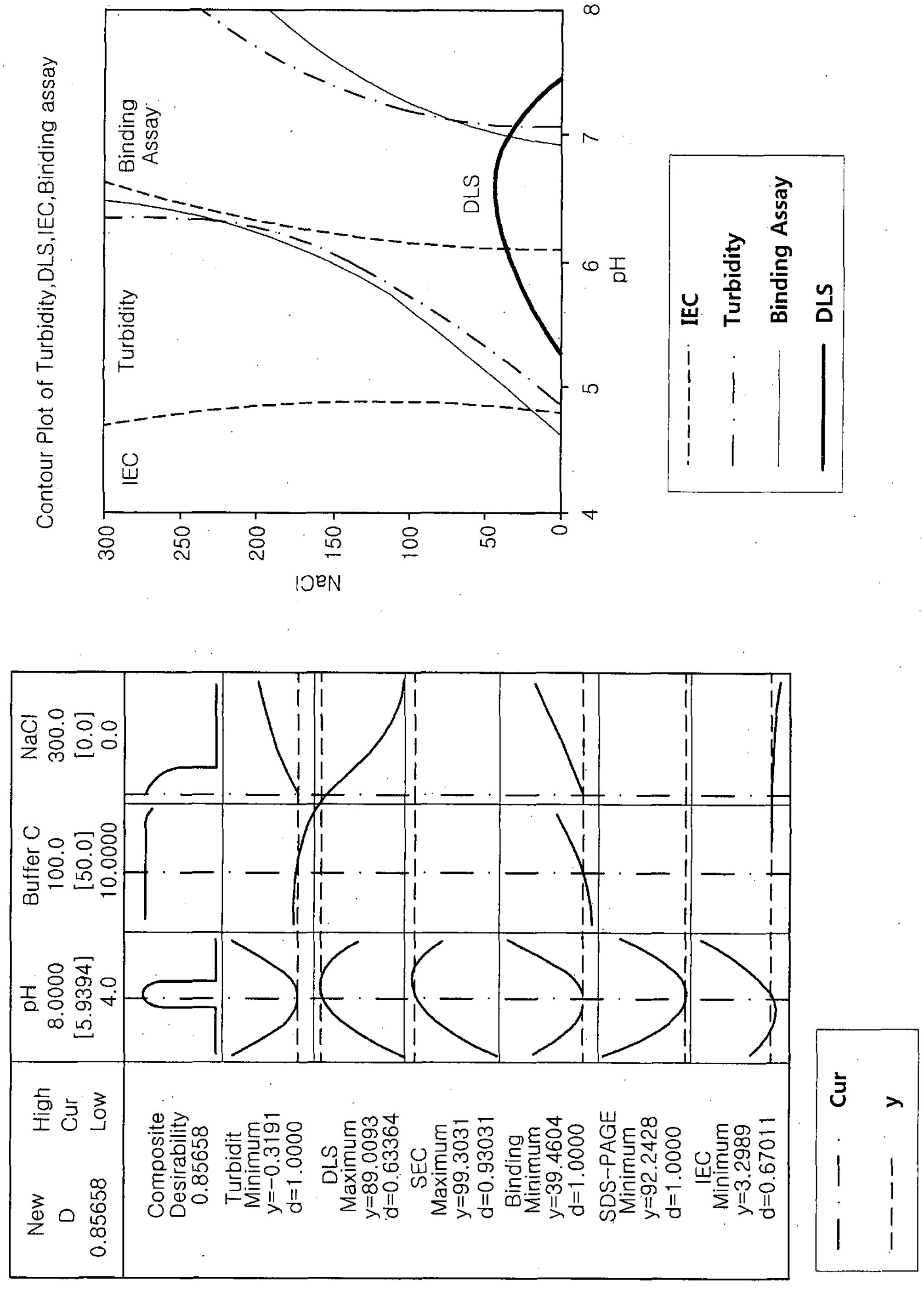
	60℃,1d, EC50 compared to #15 (ratio)
GC1118 #1	107.29
GC1118 #2	103.07
GC1118 #3	123.10
GC1118 #4	103.35
GC1118 #5	449.30
GC1118 #6	106.22
GC1118 #7	264.61
GC1118 #8	93.06
GC1118 #9	426.46
GC1118 #10	105.31
GC1118 #11	115.98
GC1118 #12	108.16
GC1118 #13	101.59
GC1118 #14	101.59
GC1118 #15	100.00
GC1118 #16	100.00
GC1118 #17	100.00
GC1118 #18	100.00
GC1118 #19	100.00
GC1118 #20	100.00

Contour Plot (using Minitab)



- Model : Quadratic
- p-value (P>F) : <0.001
- R-Square : 0.8506
- Buffer : 55mM

Fig. 19


New
D
0.85658
High
Cur
Low
pH
8.0000
[5.9394]
4.0
Buffer C
100.0
[50.0]
10.0000
NaCl
300.0
[0.0]
0.0
Composite
Desirability
0.85658
Turbidit
Minimum
y=-0.3191
d=1.0000
DLS
Maximum
y=89.0093
d=0.63364
SEC
Maximum
y=99.3031
d=0.93031
Binding
Minimum
y=39.4604
d=1.0000
SDS-PAGE
Minimum
y=92.2428
d=1.0000
IEC
Minimum
y=3.2989
d=0.67011
Cur
y
Contour Plot of Turbidity,DLS,IEC,Binding assay
NaCl
pH
IEC
Turbidity
Binding Assay
DLS
IEC
Turbidity
Binding Assay
DLS