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Investigation of Harvest Storage Condition on the Purification Process Efficiency and Structure of Human Recombinant Erythropoietin in Pilot Scale

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Abstract

The economic challenge of recombinant pharmaceutical protein production is the separation and purification process. So, optimizing the factors affecting these processes can be considered as an appropriate mechanism for improving productivity. In this research, we can find the temperature and harvest storage time as two important factors affecting the structure and amount of protein produced during the purification process. For this purpose, 7 batches were used. Four batches were kept at -20 ° C and three batches were kept at 4 °C. For this purpose, seven batches were used. The four series were kept at -20 ° C and three series kept at 3 ° C at 4 ° C. Initially, half of the volume of each batch (40 liters) was purified two months after collection, and then the remaining volume was purified after 4 months after collection. Then, the amount of eluted proteins, impurities test, SDS-PAGE and CZE was performed. The purified protein content for the four groups stored at -20 ° C was 0.51, 0.63, 0.46, 0.28 mg/ml and 0.45, 0.58, 0.37, 0.25 mg/ml after 2 and 4 months, respectively. This value was 0.34, 0.32, 0.33 mg/ml and 0.31, 0.27, 0.32 mg/ml after 2 and 4 months for three groups stored at 4 ° C. The CZE test showed that all proteins stored at -20 ° C had a glycosylated structure similar to the standard sample, but the proteins stored at 4 ° C had an incompatibility pattern after four months. The results indicate that improving the conditions of the production process can increase the process efficiency.

Key words: Human Recombinant Erythropoietin, Purification process, glycosylation pattern, harvest condition

Introduction

Protein-based drugs are approximately a quarter of the approved drugs are in the pharmaceutical industry and, given the increasing demand, have an economically valuable position (Ng et al., 2003; Tatkare, 2015). Recombinant erythropoietin is known, as one of the most prominent proteins produced in the Pharmaceutical industry. The production process of this protein is complex and time-consuming and involves two steps, which include the production of protein in the mammalian cell line and purification from impurities and contaminants over several stages (Choi et al., 2007; Jelkmann, 2011).

As a result, the protein is produced and purified under different conditions, which may affect the protein structure and thus reduce production process output. Therefore, one of the most important challenges in the manufacturing of this protein is to improve the factors affecting the production process. For this reason, various manufacturing sectors have been investigated (Saraswat et al., 2013; Hedayati et al., 2017).

The harvests are usually collected from several cell cultures and stored because the mammalian cell lines produce a small amount of recombinant protein, so some of the factors that may affect the structure of the protein are temperature and harvest time storage. The collected harvests are sometimes kept for a variable time at 4 ° or -20 ° C until the purification process is performed because the purification process is economically expensive and complex (Surabattula et al., 2011; Angela et al., 2017; Bischof and He, 2006). Due to the protein sensitivity to the physical and chemical factors, the condition of harvest preservation can alter the active pharmaceutics purified amount by affecting the protein structure (Hu et al., 2004).

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This research aimed to determine the effect of temperature and harvest storage time on qualitative and purified protein quantitative. For this purpose, seven harvests collected from cultured CHO cell lines, which were stored at two different temperatures, purified separately at two different times to investigate the effect of time interval factor in eluted protein content.

Material and Method

Sample Collection

The recombinant human erythropoietin was used as a model in this research. Briefly, recombinant EPO was produced by culture of CHO cell lines containing the gene EPO in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Grand Island, USA). After cultivation, expansion, and recovery, the supernatant was collected from the cultures. For this project, seven batches were used that produced in the manufacturing complex - Institute Pasteur of Iran. Initially, the supernatant was filtered by 0.2 µm filtration cartridge (Sartorius, Gottingen, Germany), three samples were stored at 4 ° C and four samples at 20 ° C.

In the first stage, the purification process was carried out for half volume of harvests (40 liters) within two months after production and the rest volume of samples (40 liters) purified four months after production.

The purification process of all harvest was performed industrial chromatography methods based on the recombinant erythropoietin purification protocol provided by WHO, No. 937, 2006 Appendix 4 (Organization WHO, 2006).

For this purpose, the initial and final methods of industrial chromatography were used including gel filtration chromatography, ion-exchange chromatography and high-resolution separation chromatography (K-prime, EMD Millipore, Germany). The amount of protein and bacterial content was measured at the end of each chromatography stage. Also, qualitative control tests were performed to evaluate the purified protein too.

The Procedure of the Chromatography Process

Initially, the supernatant became homogenized. Then, the samples were applied to the filtration gel chromatography column with Sephadex G-25 matrix. The column was washed with a sodium acetate buffer (PH: 5, working buffer). According to the standard protocol, the acceptable range of pH and conductivity of buffer in the G25 chromatography column is 4.8-5.2 and 3-4.5, respectively. The samples which eluted from the column were kept in a tank at 4 °C. ,

The protein content of samples was measured after homogenization and applied to the ion exchange chromatography column. These samples were washed in a Q-Sepharose ion exchange column in three steps with different buffers. First sodium acetate buffer (PH: 4) used to remove proteins that were not attached to the resin. Subsequently, sodium acetate buffer (PH:5) was used to remove proteins that were poorly bonded. At the end of this stage, the 0.3 M sodium chloride solution buffer (pH: 4.8-5.2 and conductivity 30-35 ms/cm) was used to separate the target protein from the resin. After collecting the desired protein, the total protein of the eluted samples was measured.

At the end of the chromatography steps, the obtained samples were applied to a high-resolution chromatography column with a supersex bed (G200). The sample was washed with di-sodium hydrogen phosphate buffer (pH: 6.8-7.2 and conductivity 15-18 ms/cm) and the output protein was measured.

EPO Purity Screening Tests

Qualitative control tests were performed to investigate the effect of the time interval between harvest collection and purification process on the structure of the eluted protein. The protein obtained from each chromatographic step was measured by the ELISA kit (EPO Elisa ROCHE). Subsequently, the molecular weight of the protein was measured by the SDS-PAGE method. Then, the endotoxin level, double-stranded DNA impurities, and contamination of CHO cell proteins were done for both groups of harvests. Finally, the protein isoforms pattern was evaluated by Capillary Zone Electrophoresis (CZE).

In the final stage, the erythropoietin peptide map determined by RP-HPLC. For this test, samples diluted with Tris 0.1 M to reach a concentration of 1 mg/ml. Then 25 ml of the diluted sample centrifuge with 5000 rpm for 5 minutes. After that, 0.2 ml of DI water was added and the centrifuge was done again. After desalting, 5 µg of trypsin added to samples and placed at 37 ° C for 18 h. in the end, the samples were incubated at -20 ° C to stop the enzyme reaction. After balancing the HPLC column, 50 µl of the sample and the standard were applied to the column. Samples and standard chromatograms were compared together.

Data were analyzed using the Pearson correlation test and regression analysis by using SPSS 16v IBM. Statistical results with p-value <0.05 are considered significant results.

Result

According to the standard protocol, gel filtration chromatography (G25) was performed for both groups of harvests. The amount of protein and microbial content of purified samples from this stage are as follows. (Table 1)

Then, the amount of protein purified from the ion exchange chromatography column and the microbial range of the obtained sample was investigated. The results are as follows. (Table 2)

At the end of the chromatography steps, the protein obtained from the ion exchange column was applied to the G200 column. After washing with buffer (phosphate buffer), the amount of protein was obtained and the bacterial content was examined. The results of the high-resolution chromatography are as follows. (Table 3)

In the following, the molecular weight of the proteins obtained from each chromatographic series was analyzed by SDS-PAGE method. For this purpose, the samples were reduced by the A12XReducer buffer. Then, samples were placed on the gel with standard erythropoietin alfa (35 kDa) and a molecular marker (14-115 kDa). After gel electrophoresis and fixation acetic acid, coomassie blue was used to observe the bands. The results showed all samples were in the range of 35-40 kDa. (Figure 1)

Based on the following research, the presence of two-strand DNA impurities, endotoxin levels, and contaminations of CHO cell line proteins were performed in samples. Initially, Dig DNA marking & detection kit was used to check the amount of ds-DNA impurity, the acceptable range for this test was ≤10pg / dose. The results showed all samples were in the acceptable range. (Table 4)

In the next step, the endotoxin level was measured in samples by using CAMBREX chromogenic LAL kit, the acceptable range is ≤20 IU / 100000 IU EPO. All purified proteins had acceptable endotoxin levels. (Table 4)

At the end of this step, the presence of CHO cell proteins was investigated in obtained samples. This test is important to demonstrate the purity of proteins. The standard limit for this impurity is less than 10ppm in the final eluted proteins. The results showed all samples had values below the standard for this contamination. (Table 4)

The results of the peptide map by RP-HPLC method showed that the chromatogram pattern of all samples was following the standard and acceptable samples (Figure 2)

Finally, the distribution of EPO isoforms was performed by the CZE method, to compare the glycosylation pattern of proteins in two harvest groups. At first 500 µl of samples were desalted twice with WFI by washing. Then the samples isoform pattern was compared with the isoform of standard recombinant erythropoietin (figure 3). The results showed samples that were kept at -20 ° C had an acceptable isoform pattern similar to standard sample after 2 and 4 months. Samples stored at 4 ° C had a CZE pattern similar to the standard sample, after 2 months, but the pattern of two samples didn't conform to the standard pattern after 4 months. The isoform pattern obtained from the samples in comparison with the standard sample is as follows. (Table 5)

Statistical Analysis

The results showed there is a significant difference between the time variations between Harvest collection, purification process, and chromatographic efficiency; as the interval is shorter, the amount of purified protein is higher ($P<0.01$)

Discussion

An efficient cost-effective purification process is a major challenge in the commercial production of recombinant erythropoietin to achieve pure protein with a suitable amount (Kumar et al., 2017). As a result, the manufacturing process optimization of this recombinant protein can be a great help in increasing protein production, which reduces the cost of medicines and increases the health of the community (Petrides et al., 2014).

For this reason, extensive studies have been carried out on the factors involved in the cultivation, production and purification processes.

A study by Sung et al. In 2001 examined the effect of co2 on the production of erythropoietin in the CHO cell line. The results of this research showed growth would slow down in the absence of CO2, but the amount of protein produced was 3 times higher than when

cultured in the presence of 5% CO2. While the Western blot analysis showed there is no difference in the structure of produced proteins (Yoon et al., 2001).

Another study by Ji Yong Song in 2003 examined the effects of medium temperature on the amount of production, transcriptional and heterozygous levels of erythropoietin. The culture was carried out at 37, 33 and 30°C temperature. The results showed when temperature shifted to less, the amount of production increased, but the best quality of the protein was obtained at 33°C (Yoon et al., 2003).

Another article by Kim and colleagues published in 2004 showed the effect of urea cycle enzymes were noted in the increased production of erythropoietin. They introduced ornithine transcarbamylase and carbamoyl-phosphate synthetase I enzymes into CHO cell lines with stable transfection method. In conclusion, these two enzymes increased acidic isoform production of erythropoietin by reducing the amount of ammonia present in the culture medium.(Kim et al., 2004)

In a study by Woo et al. In 2008, the temperature effect on the glycosylation pattern was investigated. The culture was carried out at 25, 28, 30, 32 and 37°C. The results showed that although the production was at the highest level at 30°C, the glycosylation pattern of protein decreased significantly with a decrease of 32°C (Ahn et al., 2008).

Other studies were conducted to optimize the production of recombinant erythropoietin in Pichia pastoris. In this field, which was written by Sukran in 2011, various pH ranges (4, 4.5, 5, 5.5 and 6) were used to determine the amount of recombinant protein production. The results showed that at optimal pH (4.5), the maximum host cell activity was associated with high production of erythropoietin, which indicates the effect of pH on protein expression (Soyaslan and Çalık, 2011).

In a study conducted by Liu et al. In 2014, increasing the production of the erythropoietin acid form in the CHO cell line was investigated by the addition of sodium butyrate. The study showed the addition of 0.5 mM butyrate resulted in a 53% decrease in extracellular sialidase activity. This enzyme is produced during the cultivation of tuberculins and leads to the reduction of the acidic form (active form) of the protein. (Liu et al., 2004)

Another study, carried out by Pankaj et al in 2015, was used to improve the recombinant erythropoietin glycosylation pattern. One of the major problems in the recombinant erythropoietin production is heterogeneity in the glycosylation pattern of producing protein. In this study, they have increased the amount of correct pattern in the produced protein by adding a GPI signal to the target protein (Singh et al., 2015).

There is a formation probability inappropriate protein structure, because of the production process of this glycoprotein occurs in the non-traditional host and under certain conditions. On the other hand, proteins are susceptible to physical and chemical agents, so changes in storage conditions can lead to change in the glycosylation structure of the protein. This section is responsible for biological and functional activity and protein stability. As a result, the time interval between harvest collections, the purification process, and temperature storage may be affected in the production process (Malachová et al., 2014; Stryjewska et al., 2013; Son et al., 2011).

The final results of this study showed the rate of production efficiency was significantly higher if we compare the 2-month interval between the collection and purification process with the samples have been purified at least 4 months after collection. On the other hand, although qualitative tests showed impurity tests are negative in both groups, but the distribution glycosylated pattern of proteins, which maintained at -20°C, is similar to the standard samples, we encountered a disproportionate distribution of the isoform pattern in the samples were kept at 4° C for 4 months. It means temperature and holding time of the harvest, effects on the quality and purification process quantity. So, procedures modeling of process purification is considered a suitable solution for increasing the purification process efficiency (Close et al., 2014; Garcel et al., 2015).

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Compliance with Ethical Standards:

- Conflict of Interest: The authors declare that they have no conflict of interest.
- This article does not contain any studies with human or animal subjects performed by any of the authors
- All authors have seen and approved the manuscript as submitted.

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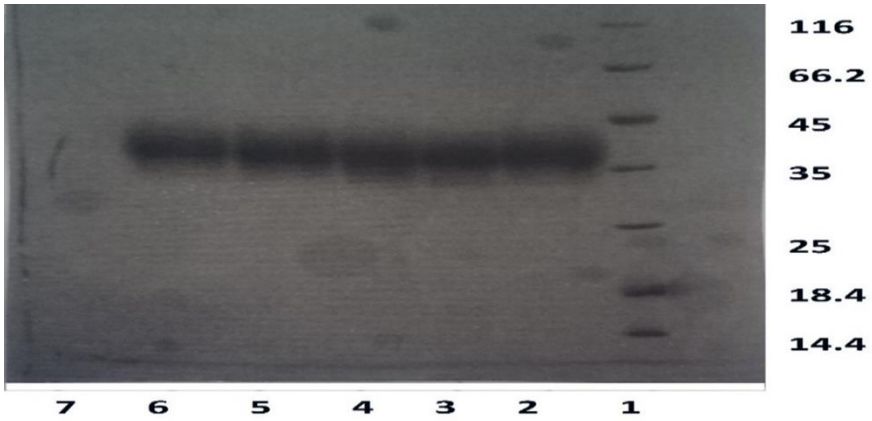

Figure 1. SDS-PAGE gel: 1: marker (14/4-116 kda) - 2: positive control (standard epo alpha) -3, 4, 5, 6: sample - 7: negative control

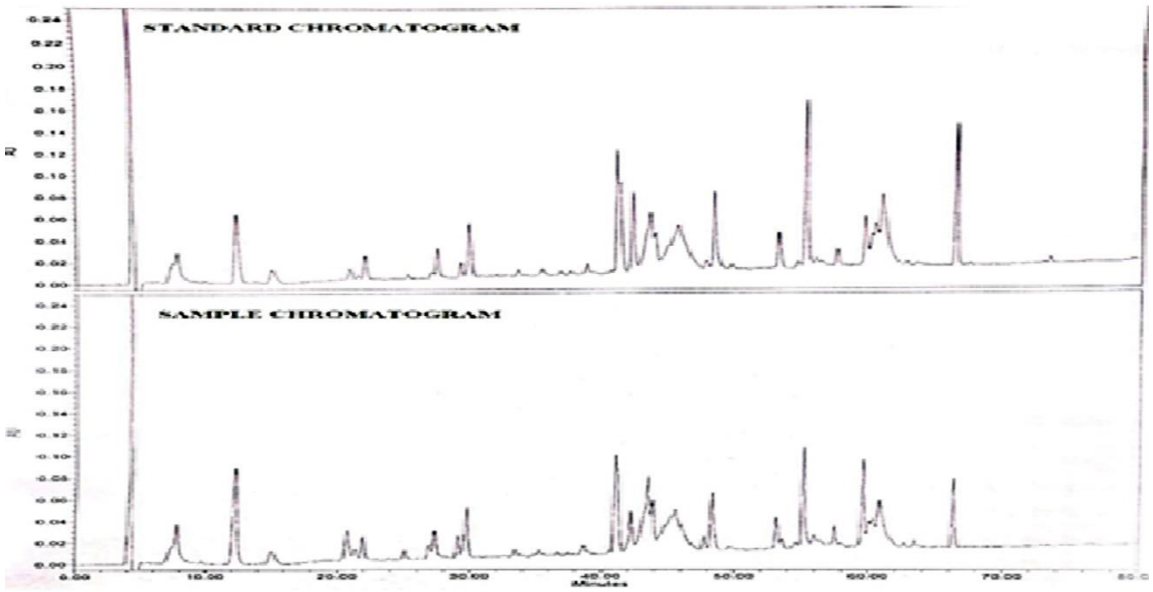

Figure 2: Comparison of erythropoietin peptide map. Up: Standard chromatogram ؛Downsample chromatogram

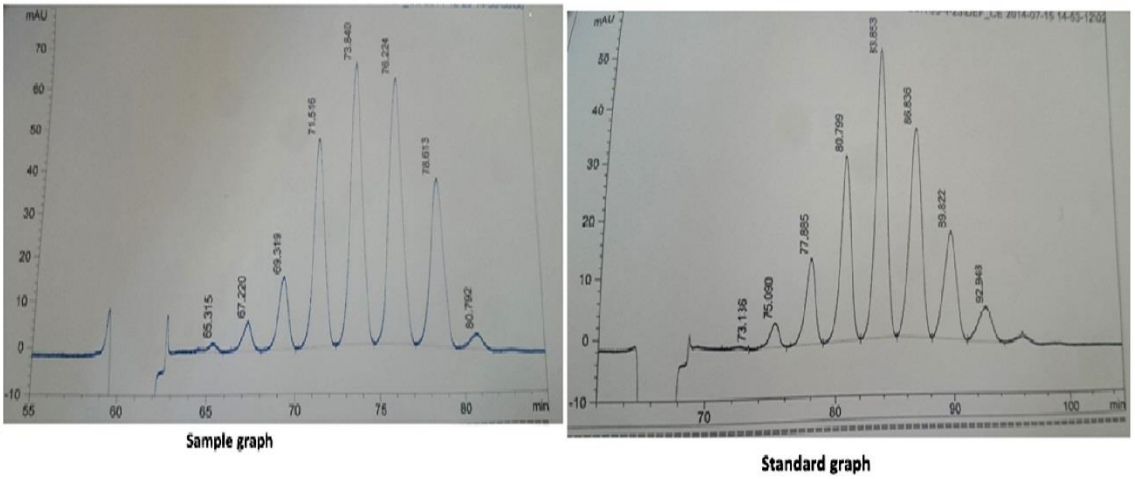

Figure 3. CZE test: comparing of isoform pattern of the sample with standard show that Isopharmic structure of the sample is acceptable

Table 1. The results of column G25: The effect of temperature and time on the content of protein and microbial mass

HARVEST (2 months after production)				
no	Storage temperature	OD of protein	total protein (mg/ml)	Microbial range
1	4^0 C	0.205	0.08	<1 CFU
2	4^0 C	0.184	0.06	<1 CFU
3	4^0 C	0.192	0.07	<1 CFU
4	-20^0 C	0.221	0.09	<1 CFU
5	-20^0 C	0.236	0.1	<1 CFU
6	-20^0 C	0.208	0.08	<1 CFU
7	-20^0 C	0.196	0.07	<1 CFU
HARVEST (4 months after production)				
8	4^0 C	0.155	0.04	<1 CFU
9	4^0 C	0.178	0.06	<1 CFU
10	4^0 C	0.147	0.03	5 CFU
11	-20^0 C	0.167	0.05	<1 CFU
12	-20^0 C	0.171	0.05	2 CFU
13	-20^0 C	0.203	0.08	<1 CFU
14	-20^0 C	0. 182	0.07	<1 CFU

Table 2. The results of the column IEX: The effect of temperature and time on the content of protein and microbial mass

HARVEST (2 months after production)				
no	Storage temperature	OD of protein	total protein (mg/ml)	Microbial range
1	4^0 C	0.247	0.23	<1 CFU
2	4^0 C	0.215	0.19	<1 CFU
3	4^0 C	0.22	0.21	<1 CFU
4	-20^0 C	0.263	0.28	<1 CFU
5	-20^0 C	0.355	0.32	<1 CFU
6	-20^0 C	0.251	0.25	<1 CFU
7	-20^0 C	0.228	0.22	<1 CFU
HARVEST (4 months after production)				
8	4^0 C	0.163	0.14	<1 CFU
9	4^0 C	0.204	0.19	20 CFU
10	4^0 C	0.158	0.14	<1 CFU
11	-20^0 C	0.172	0.15	<1 CFU
12	-20^0 C	0.193	0.17	<1 CFU
13	-20^0 C	0.23	0.21	<1 CFU
14	-20^0 C	0.207	0.19	<1 CFU

Table 3. The results of the column G200: The effect of temperature and time on the content of protein and microbial mass

HARVEST (2 months after production)				
no	Storage temperature	OD of protein	total protein (mg/ml)	Microbial range
1	4^0 C	0.311	0.34	<1 CFU
2	4^0 C	0.283	0.32	<1 CFU
3	4^0 C	0.295	0.33	<1 CFU
4	-20^0 C	0.475	0.51	<1 CFU

5	-20^0 C	0.517	0.63	<1 CFU
6	-20^0 C	0.393	0.46	<1 CFU
7	-20^0 C	0.248	0.28	<1 CFU
HARVEST (4 months after production)				
8	4^0 C	0.265	0.31	<1 CFU
9	4^0 C	0.237	0.27	<1 CFU
10	4^0 C	0.278	0.32	<1 CFU
11	-20^0 C	0.385	0.45	<1 CFU
12	-20^0 C	0.490	0.58	<1 CFU
13	-20^0 C	0.331	0.37	<1 CFU
14	-20^0 C	0.232	.025	<1 CFU

Table 4. The results of ds-DNA impurity, endotoxin level, CHO cell protein in eluted erythropoietin

sample	Ds- DNA impurity	Endotoxin level	CHO cell protein	Result
standard	≤10pg / dose	≤20 IU / 100000 IU	10 ppm	
1	2	0.3 IU	0.08	ACCEPT
2	5	0.1 IU	0.07	ACCEPT
3	3	0.1 IU	0.07	ACCEPT
4	2	0.1 IU	0.06	ACCEPT
5	2	0.5 IU	0.09	ACCEPT
6	3	0.2 IU	0.1	ACCEPT
7	2	0.1 IU	0.08	ACCEPT
8	2	0.3 IU	0.08	ACCEPT
9	2	0.2 IU	0.07	ACCEPT
10	4	0.6 IU	0.09	ACCEPT
11	2	0.1 IU	0.1	ACCEPT
12	6	0.1 IU	0.05	ACCEPT
13	7	0.4 IU	0.07	ACCEPT
14	3	0.2 IU	0.08	ACCEPT

Table 5. The results of CZE: By increasing the storage time at a sensitive temperature, it changes the protein's glycosylation form.

isoform		1	2	3	4	5	6	7	8
Acceptable content		0-15	0-15	11-20	10-35	15-40	10-35	5-25	0-15
standard		0/7	3/9	5/7	20/8	28/7	22/4	13/7	4/1
2 month after production									
1	4^0 C	0/5	6/3	8/9	17/9	27/1	14/6	19/3	5/4
2	4^0 C	2/7	3/3	15/3	17/6	23/7	17/6	16/8	3
3	4^0 C	4/5	7/32	12/4	14/18	22/35	21/74	13/26	4/25
4	-20^0 C	3/2	11/2	14/31	19/42	24/19	18/1	6/8	2/78
5	-20^0 C	1/08	4/35	15/2	21/5	26/82	13/42	11/23	6/4
6	-20^0 C	0/7	9/2	16/03	20/34	29/67	15/7	5/9	2/46
7	-20^0 C	0/5	4/35	17/21	20/08	23/24	18/41	14/31	1/9
4 month after production									

8	4^0 C	3/4	6/51	11/9	21/33	32/2	14/35	7/19	3.12
9	4^0 C	3/1	17.1	13/2	18/43	26/04	12/45	5/8	3/88
10	4^0 C	2/04	4	24.2	16/53	25/84	11/42	9/46	6/51
11	-20^0 C	1/9	4/6	11/07	14/81	30/43	16/24	16/35	4/6
12	-20^0 C	0/6	5/61	9/8	15/34	28/79	21/65	14/31	3/9
13	-20^0 C	1/3	7.64	13/4	22/05	29/32	13/65	11/22	1/42
14	-20^0 C	1/09	7/41	14/4	13/87	31/92	14/74	12/81	3/76