

An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains

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Abbreviations: cfu: Colony Forming Units;
TB: transformation buffer of CaCl₂ solution.

This paper describes an efficient bacterial transformation system that was established for the preparation of competent cells, plasmid preparation, and for the storage in bacterial stocks in our laboratory. Using this method, a number of different plasmids have been amplified for further experiments. Competent cells for bacterial transformation were prepared by the calcium chloride method with an optimum concentration of 75 mM. Three different strains of *Escherichia coli* that were tested are DH5 α , TG1 and XL1 blue, and the most efficient strain being XL1 blue. The optimal optical density (OD₆₀₀) range for competent cell preparation varied for each of the strains investigated, and for XL1 blue it was 0.15-0.45; for TG1 it was 0.2-0.5; and for DH5 α it was 0.145-0.45. The storage time of competent cells and its correlation to transformation efficiency has been studied, and the result showed that competent cells can be stored at -20°C for 7 days and at -70°C for 15 days. Three critical alterations to previous methods have been made, which

are the changing of the normal CaCl₂ solution to TB solution, the changing of the medium from LB to S.O.C., and addition of DMSO or PEG₈₀₀₀ during transformation of competent cells with plasmids. Changing the medium from LB to S.O.C., resulted in much faster growth of transformants, and the transformation efficiency was increased. Addition of DMSO or PEG₈₀₀₀ raised transformation efficiencies by 100-300 fold. Our improved bacterial transformation system can raise the transformation efficiency about 10³ times, making it becoming a highly efficient bacterial transformation system.

Plasmid transformation into bacterial competent cells is a key technique in molecular cloning. In early 1970's Cohen (Cohen et al. 1973) successfully transformed R-factor and recombinant plasmids into *E. coli* cells using a calcium chloride method. Since that time this method has been widely used due to its convenience. An alternative

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transformation method used is electroporation which results in a higher transformation efficiencies of up to 10^9 - 10^{10} transformants/ μg DNA (Ryu and Hartin, 1990). McCormac (McCormac et al. 1998) published a simple method for the production of highly competent cells of *Agrobacterium tunrefaciens/rhizobium* for transformation via electroporation. Okamoto (Okamoto et al. 1997) also reported high efficiency transformation of *Bacillus brevis* by electroporation, however, special equipment is required for electroporation that many laboratories cannot provide. Tsen has found certain strains of *E. coli* can incorporate extracellular plasmids into cytoplasm 'naturally' at low frequencies (Tsen et al. 2002). Kurien and Scofield have described a quick and moderately efficient method of bacterial colony transformation (Kurien and Scofield, 1995). More recently, Chen has proposed an alternative convenient and rapid method for the genetic transformation of *E. coli* with plasmids. By mixing the recipient cells and plasmid DNA and spreading them directly on selective medium plates containing Ca^{2+} , the so-called 'plate transformation' could achieve almost the same transformation efficiency as the classical transformation method with calcium, yet the whole protocol takes only approximately 2 min (Chen et al 2001). Based on this method, we have established an efficient system using *E. coli* competent cells for transformation plasmids. Plasmids then can be stored as bacterial stocks in our China-UK joint laboratory, which allows amplification of plasmids for future experiments.

MATERIALS

Bacterial strains

The *E. coli* DH5 α and *E. coli* TG1 were from Wuhan University (China); *E. coli* XL1 blue was from Hubei University (China) and Rothamsted Research (UK).

Plasmids

The following plasmids used in our laboratory were obtained from various sources and stored in our laboratory:

Marker gene plasmids. pUC18, pDE4, pDE108, pDE110, pCal-gus, pCal-neo, pAct1D-gus, pAHC 25, pRT99-gus, PRT99, pAHC20, pUGFP, pCX GFP.

HMW glutenin plasmids. p1Ax1, p1Dx5, p1Dy10, p1Dy12, pHMW-gus, pHMW-nos, pGAD2.

Media for bacterial growth

LB medium. 10 g/L Bacto -Tryptone, 5 g/L Bacto -Yeast Extract, 5 g/L NaCl, adjust the pH to 7.5 with NaOH autoclave to sterilize. Allow the auto-claved medium to cool to 55°C and add ampicillin (final concentration 100 $\mu\text{g}/\text{ml}$). For LB plates, 1.5% Bacto-agar (15 g/L) was added prior to autoclaving.

S.O.C. medium. 2% Tryptone (bacto), 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose.

Buffer and additional solutions

TB (CaCl_2) solution (Inoue et al. 1990). 10 mM Pipes, 55 mM MnCl_2 , 15 mM CaCl_2 , 250 mM KCl. (PIPES 3.021 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.205 g/l, KCl 18.637 g/l, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 10.885 g/l). All the components except for MnCl_2 were mixed and the pH was adjusted to 6.7 with KOH. Then, MnCl_2 was dissolved, the solution was sterilized by filtration through a pre-rinsed 0.45 μm filter unit and stored at 4°C, all salts were added as solids, always kept and used in cold.

DMSO bought from ALPHA Biotechnologies Company, LTD (SigmaD5879).

PEG₈₀₀₀ are bought from Sino-American Company (Wuhan, China), PEG₈₀₀₀ (40%) solution stored at -20°C.

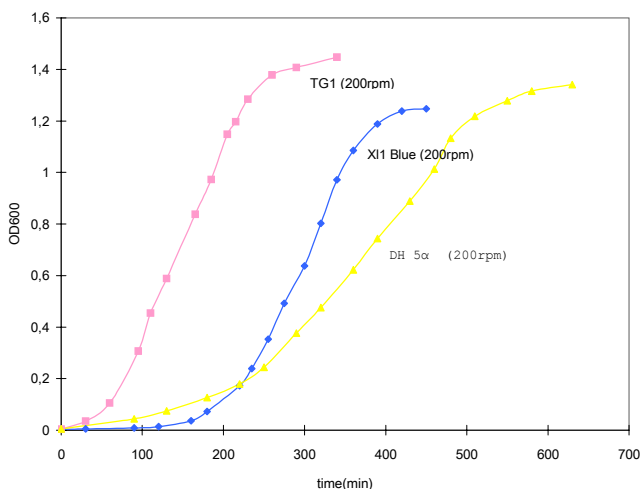


Figure 1. Growth curve of XL1 blue; TG1, and DH5 α .

METHODS

Preparation of competent cell

There are two main methods for transformation of competent bacterial cells, the calcium chloride and the electroporation method (Dargert et al. 1979; Okamoto et al. 1997; Topcu, 2000). We choose the calcium chloride method.

Calcium chloride method. A 10 μl glycerol stock of an *E. coli* strain containing no plasmids was allowed to thaw at room temperature and added to 40 ml of liquid S.O.C. media. This culture was incubated at 37°C for 1 hr, then transferred to an incubator-shaker, at 37°C, shaking at 200 rpm for 2-3 hrs until an OD₆₀₀ of 0.2-0.4 was reached. The optimum OD₆₀₀ for the different bacterial strains varied.

Table1. Transformation efficiency of plasmid DNA to different *E. coli* strains (Average).

plasmid	XL1 blue <i>E. coli</i> strains		DH5 α <i>E. coli</i> strains			TG1 <i>E. coli</i> strains			
	Normal method (Sambrook, 1989)	Quick method (Chen, 2001)	This improved method	Normal method (Sambrook 1989)	Quick method (Chen, 2001)	This improved method	Normal method (Sambrook 1989)	Quick method (Chen, 2001)	This improved method
pUC18	6.51x10 ⁶	7.1x10 ⁵	8.18x10 ⁸	2.31x10 ⁶	3.5x10 ⁵	4.15x10 ⁸	2.01x10 ⁶	3.8x10 ⁵	4.5x10 ⁸
pUGFP	7.79x10 ⁶	6.7 x10 ⁵	8.85x10 ⁸	1.66x10 ⁶	3.2 x10 ⁵	2.63x10 ⁸	1.25x10 ⁶	2.2 x10 ⁵	4.72x10 ⁸
pAHC25	6.86x10 ⁶	7.8 x10 ⁵	9.09x10 ⁸	1.56x10 ⁶	2.7 x10 ⁵	4.13x10 ⁸	1.46x10 ⁶	2.9 x10 ⁵	5.39x10 ⁸
p1Ax1	7.13x10 ⁶	6.4 x10 ⁵	8.6 x10 ⁸	1.25x10 ⁶	2.3x10 ⁵	4.52x10 ⁸	1.08x10 ⁶	2.7 x10 ⁵	4.26x10 ⁸
p1Dx5	7.09x10 ⁶	6.98x10 ⁵	8.9 x10 ⁸	1.31x10 ⁶	2.08x10 ⁵	3.99x10 ⁸	1.21x10 ⁶	2.08x10 ⁵	4.19x10 ⁸
p1Dy10	8.17x10 ⁶	6.75x10 ⁵	7.8x10 ⁸	1.20x10 ⁶	1.97x10 ⁵	3.56x10 ⁸	1.31x10 ⁶	1.97x10 ⁵	3.25x10 ⁸

Determination of their early log phase is important. The cells were pelleted by centrifugation at 8000 rpm for 1 min at 4°C, then resuspended in one-half volume (20 ml) of sterile cold TB (CaCl₂) solution, and incubated on ice for 25 min. After another centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 ml) of sterile cold TB (CaCl₂) solution to yield the final competent cell suspension. Competent cells can be stored at 4°C for up to 3 days.

Preparation of competent cells for storage as glycerol stocks. Transfer 1.6 ml of the competent cell suspension to sterile cryo-storage tubes, and add 0.4 ml of sterile 100% glycerol to give a final concentration of 20% glycerol, and then mix together. The glycerol stocks are placed at -4°C, -20°C and -70°C separately for later use.

Bacterial transformation

Plasmid transformation and antibiotic selection. Calcium chloride treatment of bacterial cells produces competent cells that will take up DNA following a heat shock step. DNA molecules, *i.e.* plasmids, which are introduced by this method, will then be replicated in the bacterial host cells. To aid the bacterial cells' recovery, the cells are incubated briefly with non-selective growth medium following the heat shock treatment. However, due to the low percentage of bacterial cells that have been transformed with the plasmid and the potential for the plasmid not to propagate itself in all daughter cells, it is necessary to select for bacterial cells that contain the plasmid. This is commonly performed using antibiotic selection.

E. coli strains such as XL₁ blue, DH5 α and TG₁ are sensitive to common antibiotics such as ampicillin. Plasmids used for the cloning and manipulating of DNA have been engineered therefore to harbour genes for antibiotic resistance to, for example, ampicillin. Thus, if following the transformation procedure, bacteria are plated onto media containing ampicillin, only bacteria that possess the plasmid DNA will have the ability to metabolize

ampicillin and form colonies. In this way, bacterial cells containing plasmid DNA can be selected.

Bacterial transformation protocol of our laboratory.

- Pre-heat plates of solid S.O.C. and S.O.C. ampicillin (final concentration 100 μ g/ml) at 37°C for 1 hr.
- Take 100 μ l competent cells and add 1 μ g/ μ l plasmid DNA 0.5 μ l.
- Add DMSO or PEG₈₀₀₀ (40%) 1 μ l.
- Incubate on ice for 30 min.
- Heat shock at 42°C for 90 s. (For an even quicker transformation method, this step can be neglect or omitted).
- Incubate on ice for 2 min.
- Add 400 μ l liquid S.O.C. medium.
- Incubate at 37°C for 45 min in an incubate-shaker.
- Spread half of the mix (50 μ l) onto a pre-heated plate with ampicillin, and the other half onto a control plate without ampicillin.
- Incubate plates at 37°C over night for S.O.C. medium (12-16 hrs).

RESULTS

Using cells in the early log phase of growth is an important factor for preparation of competent cells. By studying growth curves, the optimum OD₆₀₀ range can be determined. The growth curves of three different *E. coli* strains are shown in [Figure 1](#). Our experiment shows that the optimal optical density (OD₆₀₀) range for competent cell preparation varied for each of the strains investigated ([Figure 2](#)), and for XL₁ blue it was 0.15-0.45; for TG₁ it was 0.2-0.5; and for DH5 α it was 0.145-0.45. Another important factor is the concentration of CaCl₂. Although 50-100 mM calcium chloride can be used, but 75 mM CaCl₂ in TB solution was found to be the optimum concentration.

Calculation of transformation efficiency (colony forming units [cfu])

Transformation efficiency is defined as the number of cfu produced by 1 µg of plasmid DNA, and is measured by performing a control transformation reaction using a known quantity of DNA, then calculating the number of cfu formed per microgram DNA.

Equation for transformation efficiency (cfu/µg)

Transformant cfu = No. of bacteria colonies × dilution ratio × original transformation volume/plated volume

Example: If 21 colonies are observed on the plate, before plating, the transformed competent cells were diluted 10000 times, and the original transformation volume was 100 µl, 50 µl was used to plate, then transformant cfu is:

$$21 \times 10000 \times 100/50 = 4.2 \times 10^5$$

Transformation efficiency = Transformant cfu /plasmid DNA (µg).

If the plasmid DNA was added 0.5 µl (1µg/µl), the transformation efficiency = $4.2 \times 10^5/0.5 = 8.4 \times 10^5$ cfu/µg.

The transformation efficiency per microgram plasmid DNA to different bacterial strains is shown in [Table 1](#). These are the average data of 6 repeats, which shows the most efficient strain being XL1 blue. Our improved method can increase transformation efficiency approximately 1000 fold more than normal method (Sambrook et al. 1989), but Quick method (Chen et al. 2001) decrease transformation efficiency approximately 60-150 fold less than normal method (Sambrook et al. 1989).

The transformation efficiency of different plasmids by using different methods to different bacterial strains is shown in [Figure 3](#). We used pUC18 to set up our system, and used pUGFP, pAHC25, p1Ax1, p1Dx5, p1Dy10 to test our system, then calculated the average transformation efficiency, and got the [Figure 3](#). Then we use this improved method to amplify other plasmids such as pDE 110, pRT99,

pAHC20, pABPIgus, pRT101, pRTL2, pCX GFP, p1Dy12, pJD2, pHMW-gus, pHMW-nos, pGAD2, pGAD12 and so on, which shows that our improved method can increase transformation efficiency much more than normal method (Sambrook et al. 1989), but Quick method (Chen et al. 2001) decrease transformation efficiency obviously compared to normal method (Sambrook et al. 1989).

Effect of competent cell storage time at different temperature on transformation efficiencies

The effect of competent cell storage time at different temperature on transformation efficiencies has been studied. We use pAHC25 plasmid to transform XL1 blue competent cells, which have been stored at -4°C, -20°C, and -70°C separately for 1 hr, 1 night, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 15 days and 20 days. Normal transformation method has been used. The final result is showed in [Figure 4](#). The effect of competent cell storage time at -20°C on transformation efficiency shows XL1 blue competent cells can be stored at -20°C and used in 1 hr to 7 days without obvious decreasing of transformation efficiency, on the contrary, the transformation efficiency increased from 3 days to 7 days, then decreased gradually. [Figure 4](#) shows the effect of competent cell storage time at different temperatures on the transformation efficiency, Which shows competent cells can be stored at -20°C for 7 days and at -70°C for 15 days without losing their competency apparently.

DISCUSSION

Transformation efficiency is very important in molecular cloning experiments, and can be affected by many factors. Takahashi have reported a simple method of plasmid transformation of *E. coli* by rapid freezing (Takahashi et al. 1992). The most important being that the bacterial cells must in their early logarithmic growth period, Ryu and other authors have pointed out the importance of the early log phase for transformation (Ryu and Hartin, 1990). Bacteria that are able to take up DNA are called "competent" and competency can be induced by treatment

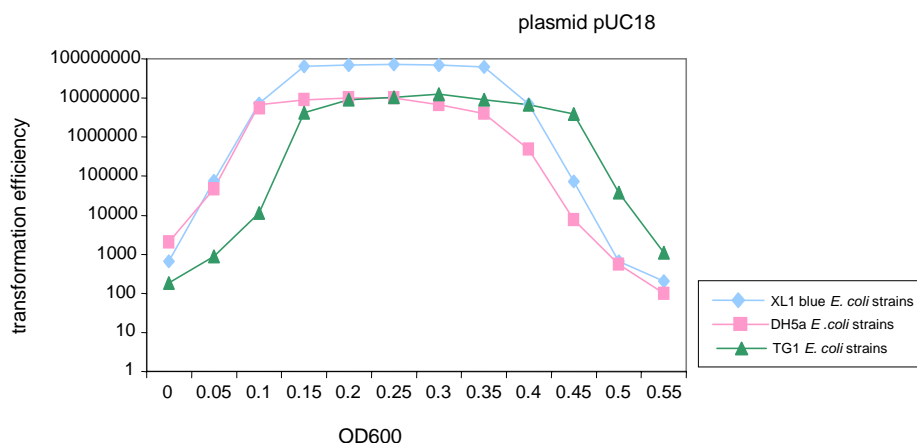


Figure 2. The optimal optical density (OD₆₀₀) range for competent cell preparation for three different strains of *Escherichia coli*. 117

with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA; the exact mechanism of this uptake is unknown. Our experiments have shown that different strains of *E. coli* have different growth characteristics, such as *E. coli*: XL1 blue, TG1 and DH5 α , therefore, the optimal OD₆₀₀ range to use for preparation of competent cells varies: For XL1 blue this is 0.15-0.45; for TG1 0.2-0.5; and for DH5 α 0.145-0.45. Competent cells prepared from the overgrowth or undergrowth bacterial cultures outside these optimal OD₆₀₀ range will have reduced or no transformation capacity. In our laboratory, XL1 blue was found to have the highest transformation efficiency; therefore it is more commonly used. Bacteria for preparation of competent cells would routinely be cultured to OD₆₀₀ = 0.2-0.4.

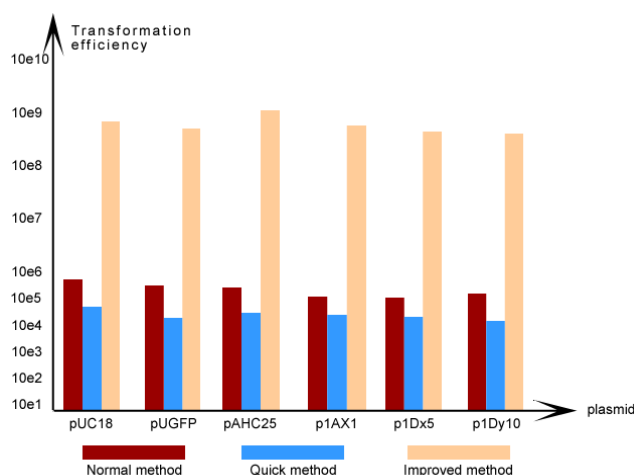


Figure 3. Transformation efficiency of different plasmids by using different method

A second factor, which can have an impact on the transformation efficiency, is that the competent cells must be maintained in cold environment, both during storage and in use. Dargert (Dargert and Ehrlich, 1979) reported that competent cells could be stored at 4°C in Calcium chloride for 24-48 hrs. In the prime 12-24 hrs, the transformation efficiency rise 3-5 times, then reduces to average level. Our experiments show that competent cells can be stored at -70°C for 15 days without obviously reducing their transformation capacity. However, if the competent cells are stored at -20°C, the highest transformation efficiencies appear at 2-7 days. But if the storage time was over 7 days, the transformation efficiency was dramatically reduced. If competent cells were stored at 4°C, they will lose their competency in only 3 days. Competent cell cannot be stored long term under liquid N₂ and cannot be defrosted more than once.

Another important factor is the concentration of CaCl₂. Although 50-100 mM calcium chloride can be used, but 75 mM CaCl₂ in TB solution was found to be the optimum concentration. Brian and Heler (Brian and Heler, 1996) first used TFB as a substitute for the traditional CaCl₂ solution. We used TB solution, which increased transformation efficiencies more than 100 fold. Using the traditional CaCl₂ method at 37°C, the no. of transformants/μg plasmid DNA was $1 \times 10^5 \sim 10 \times 10^5$. Using TB under the same conditions resulted in the no. transformants/μg plasmid DNA of $1 \times 10^7 \sim 9 \times 10^7$.

The addition of DMSO or PEG₈₀₀₀ during bacterial transformation can also affect transformation efficiency. Hanahan (Hanahan et al. 1991) found the addition of DMSO greatly increased the transformation efficiency. Similarly, incubation of competent cells and plasmid DNA in a solution of polyethylene glycol/Calcium chloride (PEG/CaCl₂) following by a brief incubation and heat shock resulted in efficient uptake of DNA (Kurien and Scofield, 1995). Our experiments show that addition of DMSO or PEG₈₀₀₀ during transformation process can give a transformation efficiency of 100-300 fold higher than the Cohen's method.

The bacterial culture medium can also affect the transformation efficiency. Jessee (Fierro, 2004; Maeda et al. 2004) suggested S.O.C. medium for growth of bacteria for preparation of competent cells. S.O.C. is a richer medium than LB medium, which therefore results in faster growth of bacteria; not only can transformants be observed sooner in S.O.C. medium after 12 hrs as opposed to 24 hrs in LB medium, but the transformation efficiency is much higher; S.O.C. giving 10-30 times higher efficiency than LB.

It is known that the effect of calcium chloride treatment can be enhanced if followed by a heating step, although there is some debate about whether the heat shock step is critical for the uptake of DNA (Chen et al. 2001; Kimoto and Taketo, 2003). When *E. coli* is subjected to a temperature of 42°C, a set of genes called the heat shock genes are expressed, which enable the bacteria to survive at such temperatures. However, at temperatures above 42°C, the bacteria's ability to uptake DNA becomes reduced, and at more extreme temperatures the bacteria will die. Although not essential, a heat shock can increase the transformation efficiency. Van der Rest (Van der Rest et al. 1999) described the use of a heat shock following electroporation to induce highly efficient transformation of wild-type *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Although Chen (Chen et al. 2001) proposed a convenient and rapid method for the genetic transformation of *Escherichia coli* with plasmids, the heat shock step was omitted and the resulting transformation efficiency is about 100 fold lower.

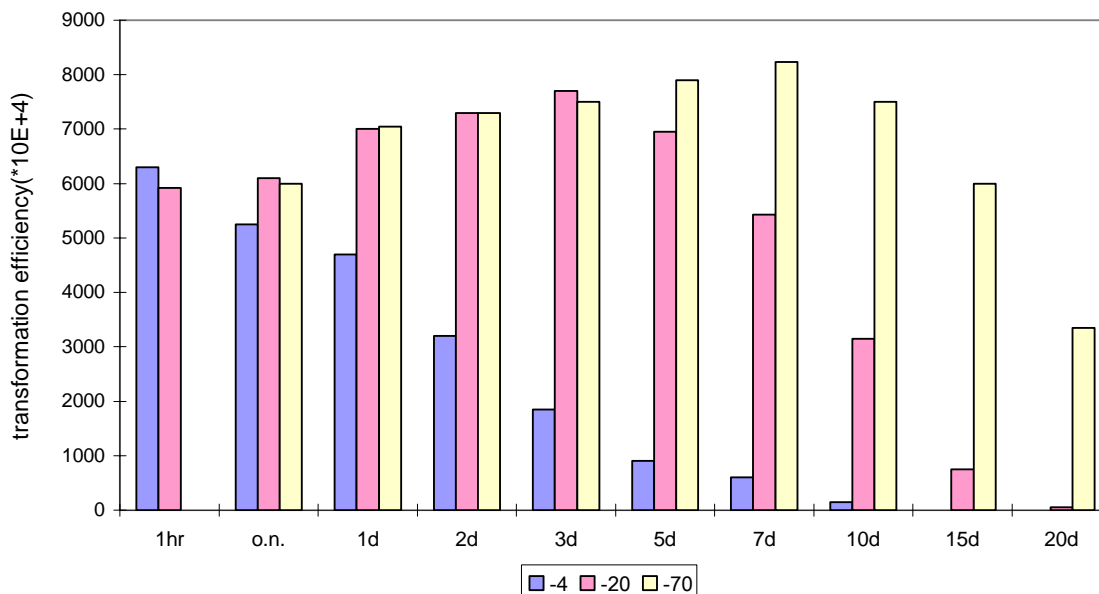


Figure 4. Effect of competent cell storage time at different temperature on transformation efficiency (XL1 blue, pAHC25, normal transformation method).

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