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Research Article

OPTIMIZED PROCEDURE FOR COMPETENT CELL PREPARATION COMPATIBLE WITH STANDARD BIOBRICK ASSEMBLY PROTOCOLS

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Summary: The primary goal of synthetic biology is to make the process of engineering biological systems easier. One of the biggest steps in this direction was the development of the BioBrick registry. It is open and contains the largest collection of standard and well characterized genetic parts worldwide. Here, we present an optimized procedure for preparation of chemical competent cells that can be used with different BioBrick parts from this registry. It is based on transformation and storage solution (TSS) procedure with some additional modifications. Our improvements include the addition of a heat shock step, the use of a different type of polyethylene glycol and the omission of the pH adjustment step. We intensively optimized all transformation conditions for the widely used general cloning strain *E. coli* DH5alfa. Our modifications resulted in a very simple, quick and cost efficient protocol that gave reliable and reproducible results with the BioBrick distribution kits. The cells were tested in a variety of applications like plasmid transformations, BioBrick assembly procedures and Aqua cloning of complex genetic constructs.

Keywords: BioBrick, competent cells, cloning, transformation.

Abbreviations: TSS – Transformation and Storage Solution; iGEM – International Genetically Engineered Machine; PEG – Polyethylene glycol; DMSO – Dimethyl sulfoxide.

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INTRODUCTION

The rapidly growing field of synthetic biology is highly dependent on fast and efficient cloning methods to build novel biological functions and systems. The BioBrick registry development greatly improved the accessibility of standard and well characterized genetic parts worldwide (Rokke et al., 2014). What remains as unsolved issue for now is finding an easy, simple and cost efficient method to generate composite genetic constructs from different parts. The major difficulty is not related to the assembly process *per se* but to the transformation step associated with it. A large number of techniques have been successfully applied to join sets of BioBrick elements in a pre-determined order but their effectiveness greatly varies depending on the quality of the used competent cells (Sleight et al., 2010). This issue affects mainly laboratories that cannot afford commercial competent cells due either to their significant cost or to the fact that companies do not deliver these products to some regions. In these cases researchers have to prepare their own competent cells. Many protocols for home made competent cells have been published but most of them require high-purity grade chemicals, long and complex experimental setups and/or specialized equipment like electroporation devices (Sambrook and Russell, 2006). To allow everyone to have an equal chance to use standard BioBrick parts for their synthetic biology projects, we need a new protocol for competent cells with several unique characteristics. First of all it should be simple, reliable and safe enough that even student teams in one of the popular synthetic biology competitions (like iGEM for example) could use it without supervision. Another very important matter is the price. To popularize synthetic biology in developing countries, the competent cells should be as cheap as possible. Last but not least is the time efficiency of the procedure. In some cases deep freezers that can support -80°C are not available and then fresh competent cells should be prepared prior to each cloning procedure. When such limitation is present the time cost of the protocol is maybe the most important component.

In this work, we present a simple, fast and cost-efficient method for generation of competent *E. coli* cells. It is based on the transformation and storage solution (TSS) procedure which has been intensively modified and simplified (Chung et al., 1989). No dangerous chemicals are required and the hands-on time is reduced compared to other methods. In addition, we successfully used competent cells prepared according to our protocol in different cloning scenarios involving BioBrick parts.

MATERIALS AND METHODS

The competent cells were prepared and transformed as follows: 50 ml of liquid LB medium (Miller‘s LB Broth Base (ThermoScientific) containing 10 g SELECT Peptone 140 + 5 g SELECT Yeast Extract + 10 g sodium chloride per liter) was inoculated with 500 µl overnight culture of *E. coli* in a 500 ml Erlenmeyer flask. After that cells were cultured at 37°C with shaking until the optical density of the culture reached values in the range of 0.3 to 0.4. In the meantime, 1x TSS

buffer was prepared. It contained 2 g PEG 6000, 0.3 ml 1M $MgCl_2$, 0.3 ml 1M $MgSO_4$ and LB media to a final volume of 19 ml. All compounds were mixed until PEG was dissolved. The mixture was then filter sterilized (0.22 µm filter) and 1 ml DMSO was added. The TSS buffer can be stored at -20°C for a few months. Once the proper optical density was achieved, 50 ml of the culture were transferred into one 50 ml Falcon Conical Centrifuge Tube and centrifuged at 2,700 x *g* for 10 min at 2-4°C. The resulting cell pellet was dissolved in 1 ml of pre-chilled 1x TSS buffer with gentle pipetting. 100 µl aliquots of TSS suspended cells were then transferred to labeled and pre-chilled 1.5 ml Eppendorf tubes while ensuring the cells remain well mixed and cold. Cells can be used immediately or stored at -80°C. When performing transformation, an aliquot of competent cells was thawed on ice, DNA was added (not more than 1/10 of the cell volume) and this mixture was incubated for 30 min on ice. After that cells were heat shocked for 45 s at 42°C and next kept for 2 min on ice. Finally 900 µl room temperature SOC medium (S.O.C. Medium (Invitrogen) containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to each vial followed by incubation for 1 h at 37°C with shaking. Finally appropriate amounts were spread onto a prewarmed (37°C) LB plate with antibiotic.

All optimization steps in our protocol were performed using the *E coli* strain DH5α. Stock solution of pSB1C3 (100 pg/µl) was used as control to determine the transformation efficiency. Standard restriction and ligation techniques were used for BioBrick parts assembly according to the BioBrick Protocol (Rokke et al., 2014). Aqua cloning was also performed as previously described (Beyer et al., 2015). The only difference in that case was that we used Top 10 strain instead of DH5α for competent cells preparation. No additional optimizations were made for the Top 10 strain. All primers used for PCR amplification of parts and vectors are listed in Table 1.

RESULTS

We optimized the standard TSS preparation method for one of the most intensively used stains in molecular cloning applications – DH5α. Initially we checked the importance of the PEG polymer size used in TSS buffer recipes.

Table 1. Oligos used in BioBrick amplification and Aqua cloning procedures.

Oligo	Sequence	Purpose
VF2	TGCCACCTGACGTCTAAGAA	BioBrick amplification
VR	ATTACCGCCTTTGAGTGAGC	BioBrick amplification
AmilCP_CvF	TATTCTAGATTGACAGCTAGCTCAGTCCT	Aqua cloning
AmilCP_CvR	TATCTGCAGTTATTAGGCGACCACAGGTTTG	Aqua cloning
Assembly_vectorF	ATCAGCTCACTCAAAGGCGGTAAT	Aqua cloning
Assembly_vectorR	TGGTTTCTTAGACGTCAGGTGGCA	Aqua cloning

Our test transformations confirmed previous data that the PEG size has little to no influence over the transformation efficiency (Cui and Shearwin, 2017). Thus, in all following TSS preparations we used PEG 6000. Next, we checked pH of the TSS solution. Despite numerous reports indicating the value of 6.5 as the optimal pH (Chung et al., 1989), the TSS solution worked fine without need of pH adjustment. We observed a difference of extra 4.57% in the total colonies number when a pH adjusted TSS solution was used (sum of 3 replicates, one of which showed more colonies with a non-adjusted TSS solution). This value was in the range of the experimental mistake indicating that the pH calibration step can be omitted. Most protocols for home-made competent cells need a start culture with optical density in a narrow range to produce good yield. In our tests, values within the range of 0.3 - 0.4 gave competent cells with higher efficiencies (up to 2.10^7 with 300 pg pSB1C3) compared to the optical densities of 0.5 – 0.6 (9.10^6 – 1.10^7 with 300 pg pSB1C3). In contrast to other protocols, competent cells with sufficient quality for a successful BioBrick assembly procedure (tested with restriction/ligation and Aqua cloning assembly of chromoprotein expression cassettes in pSB1C3 with J23102 constitutive promoter) could be obtained using cultures with optical densities up to 0.8 - 0.9 (Chan et al., 2013). Next, we optimized the parameters of the transformation procedure. The original TSS protocol claims that a heat pulse is not necessary and the incubation time at 4°C is not crucial, so there are no critical timing steps. Our results confirmed that incubation time on ice was not essential. In general, 30 min turned to be a reasonable time interval to obtain optimal efficiency in terms of colony number and time invested. Nevertheless, the heat pulse had an essential role in our experiments. Without it we detected a significant reduction in the transformants number (approximately two orders of magnitude). The time of the pulse was intensively optimized in a series of experiments. Among all values tested (30 s, 45 s, 60 s, 75 s, 90 s and 120 s), the most optimal one was found to be 45 s at 42°C. With all these adjustments we routinely obtained cells with efficiency of 1-2 x 10^7 when tested with 300 pg standard vector pSB1C3.

Further on, we tested the competent cells in real cloning scenarios with sets of BioBrick parts. First we used a regular BioBrick assembly protocol to clone a blue AmilCP chromoprotein producing part into the standard assembly vector pSB1A3. Ligation mixtures were transformed in the competent cells and successful transformants were identified based on correct color development. After isolating a significant number of correct transformants, we further tested this assembly procedure using different chromoprotein expression cassettes and assembly vectors with varying antibiotic resistance including chloramphenicol, kanamycin, ampicillin and tetracycline. Our results indicated that the efficiency of the competent cells was absolutely sufficient to join together two parts of BioBrick components. Having in mind the importance of the scarless cloning methods, in some cases we decided to use the cells in a restriction and ligation free cloning procedure. We selected Aqua cloning since it is one of the

cheapest and fastest methods of this type published so far. We performed an Aqua cloning reaction with two fragments – a BioBrick vector pSB1A3 and an AmilCP expression cassette. Both fragments were PCR amplified (primers are given in Table 1) in a way to have 24 base pairs terminal homology to each other and then the Aqua cloning procedure was carried out as previously published (Beyer et al., 2015). In this case we used strain Top 10 for competent cells preparation since it was reported to be significantly more efficient than DH5α for this type of cloning. Our experiment was successful as a large number of positive clones with a strong blue color were identified indicating that the Aqua cloning procedure worked as expected and these cells can be used.

Summarizing, our current protocol is simpler than the original version for TSS competent cell preparation. The modified procedure described in this work has no limitations for the type of PEG used and does not need a pH adjustment of TSS like its predecessor. Moreover, it relies on arguably the most intensively used *E. coli* cloning strain at the moment – DH5α. All optimization steps and proof of principle cloning experiments were performed with standard BioBrick vectors and fragments suggesting that our competent cells can be used with the distribution kits from the Standard Registry of Biological Parts without any further modifications.

DISCUSSION

The quality of the used competent cells is of critical importance when using BioBrick fragments together in a composite construct. Undoubtedly the best choice from experimental point of view is to buy some highly efficient competent cells distributed by different companies. Unfortunately, their price is quite high and moreover, the companies do not deliver these products to some geographic locations. Because of these reasons a large group of scientists worldwide are enforced to prepare their own competent cells. To facilitate this process, here we propose a modified version of the TSS protocol that allows simple, one-step preparation of competent *E. coli* cells (Chung et al., 1989). The TSS procedure is among the most used ones for generation of homemade competent cells. Despite its numerous advantages, the original protocol was never systematically tested before with constructs from the BioBrick distribution kits. All fragments used in this work were in the pSB1C3 vector backbone with chloramphenicol resistance and their concentrations were relatively low (200-300 pg/μl). Moreover, these pDNAs were not fresh isolation preps and can suffer some damage due to different transportation problems. All these combined make the BioBrick part transformation not so easy as the regular transformation with pUC19 plasmid. To guarantee the success of this process, here we propose an optimized TSS-based procedure for competent cells preparation compatible with all standard BioBrick assembly protocols.

Our experiments proved that the TSS buffer did not need a pH adjustment. The thermal pulse at 42°C turned to be crucial for high efficient transformation of the general cloning strain DH5α. It should be noted that the TSS procedure eliminates many centrifugation, washing, and time

consuming incubation steps of other current methods like calcium chloride treatment. Our modified version is fully tested with BioBrick parts and has some additional advantages compared to the original. It is independent of the type of PEG used and the pH adjustment step is removed – important point for small iGEM teams with no access to a calibrated pH-meter. Moreover, all optimizations are done with the common DH5alfa strain which is easy to obtain and use. In addition, our protocol (just like the original TSS procedure) can tolerate start cultures with more varying optical density than other available methods. All these advantages make our modified protocol extremely useful for beginners in the field of synthetic biology or in case one needs to prepare competent cells routinely due to storage limitations. It is easier, cheaper and faster compared to the Hanahan protocol (using CCMB80 buffer) used by Tom Knight and currently recommended by the Registry of Standard Biological Parts (http://parts.igem.org/Help:Protocols/Competent_Cells). Furthermore, our panel of test with different BioBrick parts and cloning techniques proved that all prepared competent cells were of sufficient quality to be used in every regular cloning scenario with these types of DNA fragments.

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