Indian Journal of Biochemistry & Biophysics
Vol. 46, October 2009, pp. 395-400

Analysis of comparative efficiencies of different transformation methods of *E. coli* using two common plasmid vectors

Aryadeep Roychoudhury[*1], Supratim Basu[2] and Dibyendu N Sengupta[2]

[1]Department of Botany, Plant Molecular Biology and Biotechnology Laboratory, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India
[2]Department of Botany, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Kolkata 700009, West Bengal, India

Received 13 April 2009; revised 04 September 2009

The efficiencies of different transformation methods of *E. coli* DH5$_\alpha$ strain, induced by several cations like Mg^{2+}, Mn^{2+}, Rb^+ and especially Ca^{2+}, with or without polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) were compared using the two commonly used plasmid vectors pCAMBIA1201 and pBI121. The widely used calcium chloride ($CaCl_2$) method appeared to be the most efficient procedure, while rubidium chloride (RbCl) method was the least effective. The improvements in the classical $CaCl_2$ method were found to further augment the transformation efficiency $(TR)_E$ for both the vectors like repeated alternate cycles of heat shock, followed by immediate cold, at least up to the third cycle; replacement of the heat shock step by a single microwave pulse and even more by double microwave treatment and administration of combined heat shock-microwave treatments. The pre-treatment of $CaCl_2$-competent cells with 5% (v/v) ethanol, accompanied by single heat shock also triggered the $(TR)_E$, which was further enhanced, when combined heat shock-microwave was applied. The minor alterations or improved approaches in $CaCl_2$ method suggested in the present study may thus find use in more efficient *E. coli* transformation.

Keywords: Calcium chloride, *E. coli*, Ethanol, Heat shock, Microwave, Plasmid DNA, Transformation efficiency

Transformation in genetics is defined as the uptake, introduction and expression of genetically engineered or naturally occurring foreign DNA by cells. It generally involves the following stages: adsorption and binding of extracellular DNA to the cell surface at 0°C in the presence of high concentration of cation(s); subsequent entry of DNA across the wall-membrane complex into the cell cytosol by a heat pulse from 0°C to 42°C, during which the DNA becomes insensitive to DNAse and is presumably transported to a site within the periplasm or inside the inner membrane; and finally, the internalised DNA is established either by forming a stable replicon itself or by homologous recombination with a resident replicon[1,2].

The bacterial transformation occurs naturally in many genera, such as *Micrococcus*, *Haemophilus, Bacillus and Streptococcus* having proteins on their exterior surface that can bind to DNA and transport it internally. However, it is still a rare event for most bacteria, including *E. coli* to naturally uptake foreign DNA from the environment[3,4]. Much more DNA is bound to the outside of cells than is actually taken up, so that DNA transport across the cell envelope rather than the external binding is a limiting step in producing transformants. The success of *E. coli* to uptake phage, chromosomal or plasmid DNA can be achieved only when the recipient cells are made "competent" through induction by certain artificial conditions, especially by treatment with any of the cation(s) like Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+} or Rb^+ at 0°C, followed by brief incubation at 42°C[5-7], when 0.1 to 1% of the tightly bound DNA becomes resistant to DNAse[2].

The transformation efficiency $(TR)_E$ is a quantitative number that represents the total number of bacterial cells, expressing the antibiotic resistance marker divided by the amount of DNA used in the experiment. $(TR)_E$ = {Number of transformants μg^{-1} of DNA} × {Final volume at recovery (ml)/volume plated (ml)}. It represents the total number of bacterial cells transformed μg^{-1} of DNA. In practice, smaller amounts of DNA are used (5-100 ng), since too much of DNA inhibit transformation process.

The earlier report has demonstrated that bacteria treated with ice-cold solutions of $CaCl_2$ and then briefly heated to 42°C could be transfected with

*Corresponding author
Ph: 91-033-2462 8040
E-mail: aryadeep.rc@gmail.com

bacteriophage λ DNA[7]. The heat shock releases an enzyme β-lactamase from the periplasm to the extracellular environment, indicating membrane disintegration. Subsequently, it was shown that the same method could be used to make the cells susceptible to uptake of plasmid DNA[6] and bacterial chromosomal DNA[8]. Since these initial studies, several factors have been elucidated that cause an increase in $(TR)_E$ like addition of multiple cations in the transformation mixture[9] and treatment of bacteria with dimethyl sulfoxide (DMSO) in the presence of both monovalent and divalent cations[5,10] or using polyethylene glycol (PEG) for the preparation of competent cells[11].

A very simple one-step method of preparation of competent *E. coli*, without applying heat shock and subjecting to a very short incubation time on ice has also been proposed[12]. Another highly reproducible method is reported, where the bacterial culture is grown at a much lower temperature (18-23°C) rather than the conventional 37°C[13]. Rubidium ions (RbCl) can also be used for transformation[14]. The short heat-pulse (42°C) step that facilitates DNA entry into the cells by lowering the membrane potential in standard transformation procedure has been replaced by incubating the DNA-adsorbed competent cells with any chemical that reduces membrane potential[15].

The transformation being a membrane-bound phenomenon is most likely to be influenced by the well-known membrane-perturbant ethanol, which leaches lipopolysaccharides (LPS) from competent *E. coli* surface and affects DNA adsorption to the cell surface[16]. However, $(TR)_E$ decreases gradually with increase in ethanol concentration. Substitution of 42°C by a microwave pulse at the lowest setting power (180 W, 1 min) or subsequently exposing the 42°C heat-shocked cells to microwave treatment also significantly improves *E. coli* transformation[17]. However, higher exposition time or augmented microwave power steadily lowers or reduces the higher efficiency.

In the present study, we have compared the efficiency of some of the methods of *E. coli* transformation using two common plasmid vectors the ~14.7 Kbp pBI121 and ~11.9 Kbp pCAMBIA1201. Since *E. coli* does not undergo spontaneous or natural transformation, we have attempted to find out which method fits best to induce the greatest competence, so as to give the maximum $(TR)_E$ of the above two vectors.

Materials and Methods

Bacterial strain, plasmids and growth conditions

The *E. coli* cells of $DH5_\alpha$ strain, a genetically engineered and highly transformable strain was purchased from Clonetech, USA and used for transformation. The vector pBI121 was obtained as a kind gift from Prof. Sudhir K Sopory, ICGEB, New Delhi, while the other vector pCAMBIA1201 was from Dr. S K Raina, IARI, New Delhi. The pBI121 has a low copy RK2 origin of replication and contains neomycin phosphotransferase (*nptII*) gene that codes for resistance to kanamycin. The pCAMBIA1201 is a derivative of pVS1 origin which replicates to 7-10 per cell[18]. This plasmid contains chloram phenicol acetyltransferase (*cat*) gene that codes for chloramphenicol resistance. The bacterial cells were grown overnight in Luria-Bertani (LB) liquid medium at 37°C, except in SOB medium (Protocol D), where the culture was grown at 23°C.

Protocol A: $CaCl_2$ method of transformation with modifications

The *E. coli* cells were grown to log phase (up to OD_{600} = 0.1, i.e., 5×10^7 cells ml^{-1}), the competent cells prepared using ice-cold, 0.1 M $CaCl_2$ and the transformation carried out according to the standard protocol[7]. We also carried out various minor modifications in this standard transformation process. The entire scheme is listed as follows: *Protocol 1*: Single heat shock, followed by cold treatment (90 s-42°C/15 min-0°C); *Protocol 2*: Single microwave treatment, followed by cold shock (1 min-180 Watt/15 min- 0°C); *Protocol 3*: Double alternate cycles of heat and cold shock; *Protocol 4*: Double alternate cycles of microwave treatment and cold shock; *Protocol 5*: Triple alternate cycles of heat and cold shock; *Protocol 6*: Single heat shock combined with single microwave treatment [(90 s-42°C/15 min-0°C) + (1 min-180 Watt/15 min-0°C]; *Protocol 7*: Single heat shock followed by cold treatment applied on $CaCl_2$-competent cells, pre-treated with chilled 5% (v/v) ethanol for 30 min at 0°C, followed by ethanol removal by centrifugation; *Protocol 8*: Single heat shock combined with single microwave treatment applied on $CaCl_2$-competent cells, pre-treated with chilled 5% (v/v) ethanol for 30 min at 0°C, followed by ethanol removal by centrifugation; and *Protocol 9*: Single heat shock step substituted with 10% (v/v) ethanol treatment, followed by incubation at 0°C for 30 min

In each of the protocols, 0.1 µg of either of the two plasmids was added to 0.2 ml of competent cell suspension for transformation. About 0.1 ml of cells from each set were plated (in triplicates) in presence of the corresponding antibiotics, kanamycin (50 µg ml^{-1}) for pBI121 and chloramphenicol (34 µg ml^{-1}) for pCAMBIA1201 and the plates incubated at 37°C for 17-20 h to obtain the transformant colonies.

Protocol B: One-step method of competent cell preparation

The *E. coli* cells were grown to the early exponential phase (up to OD_{600} = 0.3 – 0.4) and the cells made competent using ice-cold 1 × TSS buffer [LB broth containing 10% (w/v) PEG 3350, 5% (v/v) DMSO and 50 mM Mg^{2+} ($MgSO_4$) at a final pH of 6.5], following the standard protocol[12]. Transformation and plating were done as described in Protocol A.

Protocol C: Competent cell preparation using rubidium chloride (RbCl)

The cells from overnight culture were grown up to OD_{550} = 0.48 and made competent, following treatment first with ice-cold buffer 1 [30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2.H_2O$, 50 mM $MnCl_2.4H_2O$ and 15% (v/v) glycerol] and then with ice-cold buffer 2 [10 mM MOPS-KOH, pH 6.5, 10 mM RbCl, 75 mM $CaCl_2.H_2O$ and 15% (v/v) glycerol] in 5:1 ratio[14]. The subsequent steps of transformation and plating were similar to Protocol A.

Protocol D: Competent cell preparation using Inoue method

The cells from the overnight culture were grown in SOB medium [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, and 2.5 mM KCl; after autoclaving, 20 mM $MgSO_4$ was added] at 23°C until OD_{600} = 0.4-0.6. The cells were made competent, following treatment, first with ice-cold transformation buffer (TB) [10 mM HEPES, 250 mM KCl, 15 mM $CaCl_2$, all the components except $MnCl_2$ were added and pH adjusted to 6.7 with KOH, finally 55 mM $MnCl_2$ was dissolved] and then with ice-cold TB, supplemented with 7% (v/v) DMSO[13] in 4:1 ratio. The subsequent steps of transformation and plating were again similar to Protocol A.

Isolation and purification of plasmid DNA for confirmation

Plasmid DNA was isolated from the transformed *E. coli* cells by the method of alkaline lysis with SDS[19] and purified by the method of polyethylene glycol (PEG). The purity of plasmid DNA was checked by measuring its $(Absorbance)_{260}$/$(Absorbance)_{280}$ ratio, which mostly attained the value of about 1.8-1.9 in our case. Finally, the purified plasmids were visualised through electrophoresis in 1% (w/v) agarose gel (data not shown). Thus, the transformants were screened for the presence of plasmid DNA and the $(TR)_E$ was finally calculated.

Statistical analysis

All the experimental data values were means from three independent experiments (i.e., conducted thrice), each experimental set was done with three replicate plates for each protocol and the results presented as mean ± standard error (SE). The significance of differences (increase and decrease) between the mean values was statistically evaluated by two-sided Student's t-test using the Windows 98/Microsoft Excel 97 computer package for significance. The statistical significances were all calculated at $P < 0.01$.

Results and Discussion

Although *E. coli* has developed into a universal host organism both for molecular cloning and for a diverse set of assays involving cloned genes, the technique of *E. coli* transformation is highly inefficient even using competent cells. Chemical transformation and electroporation are the two methods used to transform *E. coli* cells with plasmid DNA. Electroporation provides transformation efficiencies at least 10-folds greater than chemical methods, but it requires the electroporation apparatus that is highly expensive. Commercially available, highly efficient competent cells are also costly and are to be kept in a frozen condition at -80°C. So, the laboratory which cannot afford these options, the classical methods using various cations in the transformation buffer and a short heat pulse at 42°C is the usual choice.

The variations in the optimized $(TR)_E$ of several protocols depend on the nature of cations in different buffers, treating the cells with reducing agents, harvesting the cells at specific stages of growth cycle, altering the temperature of the growth of culture before exposure to chemicals, extent and temperature of heat shock, freezing and thawing cells etc. Interaction of externally added DNA with intact cells is highly ion-specific, with the several cations promoting different levels of binding to the cells. However, even after generation of competence by

these methods, it is often found that the $(TR)_E$ is quite low. Either the vast majority of DNA molecules will not enter any cell or the majority of bacterial cells will receive no DNA at all. Report suggests that only 1-2% of the cells could be transformed. Approximately only one in every 10,000 cells successfully incorporates the DNA. So, it is essential not only to test the most powerful protocol, but also to carry out several additional improvements of the optimized protocol or technique, so as to obtain better competent cells or derive higher $(TR)_E$. The improvements in transformation frequency are attributed to the power of empirical experimentation.

Comparative efficiencies of standard methods of transformation with the two plasmids

Several proposed methods of competent cell preparation have been reported earlier. The widely used method for DNA uptake involves suspending bacterial cells in ice-cold, 0.1 M $CaCl_2$ and applying a brief heat shock[7,20]. In another high efficiency method[13], the standard transformation buffer (0.1 M $CaCl_2$) was replaced by a buffer containing more cations, such as 15 mM $CaCl_2$, 55 mM $MnCl_2$, 250 mM KCl and 10 mM [Piperazine-N, N'-bis (2-ethanesulfonic acid)] i.e., PIPES, pH 7.6. Again, RbCl has also been used in association with $CaCl_2$, $MnCl_2$, potassium acetate and glycerol[14]. Initial experiments also indicated that the bacterial cell competence could be induced by the presence of PEG, DMSO and several cations. Based on this, a simpler method of one-step preparation of competent cells was devised[12]. Thus, we first compared the efficiency of different standard methods of bacterial transformation employing Mg^{2+}, Mn^{2+}, Rb^+, DMSO, PEG etc together with the most commonly used Ca^{2+} (Table 1).

The two plasmid vectors used for our analysis were pBI121 and pCAMBIA1201. The observed $(TR)_E$ can be summarized as follows: For pCAMBIA1201, $CaCl_2$ method (protocol A) > Inoue method (protocol D) > one-step method (protocol B) > RbCl method (protocol C). Compared to $CaCl_2$ method, RbCl method was 20-folds less efficient, while direct method and Inoue methods were 1.75-folds and 1.3-folds less efficient respectively; in case of pBI121, $CaCl_2$ method (protocol A)>one-step method (protocol B)>Inoue method (protocol D)>RbCl method (protocol C). The RbCl, Inoue and one-step methods were respectively 32-folds, 13-folds and 3.2-folds less efficient than the classical $CaCl_2$ method. So, for both the plasmids, RbCl method proved to be the least efficient, while $CaCl_2$ method yielded the maximum and much higher number of transformants.

Table 1—Analysis of the TR_E with different standard methods [TR_E expressed as {Number of transformants μg^{-1} of DNA} × {Final volume at recovery (ml)/volume plated (ml)}. The data are represented as $(TR_E) \times 10^3$. The values represent mean ± SE of triplicate plates for each experimental set. Each experiment is repeated thrice with similar results]

Methods	pBI121	pCAMBIA1201
Competent cell preparation using RbCl (Protocol C)[14]	0.387 ± 0.018	0.687 ± 0.036
Inoue method (Protocol D)[11]	0.965 ± 0.098	10.500 ± 0.110
One step procedure (Protocol B)[10]	3.842 ± 0.260	7.962 ± 0.310
Competent cell preparation using $CaCl_2$ (Protocol A)[3]	12.440 ± 0.500	14.000 ± 0.600

Data are found statistically significant at $P<0.01$ (Two-way analysis of variance)

It is known that the different cations alter the stability of DNA duplex, induce DNA aggregation through cross-linking of DNA strands and mediates binding between DNA and LPS receptor molecules on cell surface[16,21]. Our data showed better efficacy of Ca^{2+} alone in inducing the uptake of both pBI121 and pCAMBIA1201, thus supporting the earlier propositions that greater availability of Ca^{2+} leads to the greater bridging up of DNA and LPS into DNA-LPS complex, which is the key step in foreign DNA entry[22-24].

Comparative efficiencies of different modified-$CaCl_2$ transformation methods with two plasmids

We have experimentally confirmed that for 0.2 ml of $CaCl_2$-competent cells, 0.1 µg of DNA and 90 s of 42°C heat shock gave the best $(TR)_E$, compared to the other transformation protocols. Earlier reports suggest that the routine $(TR)_E$ with $CaCl_2$ method are at least 10-folds lower, when the input DNA is from ligation reactions, sensibly reducing the number of recombinant clones obtained per plate. So, our next objective was whether any additional improvement or modification of this method could augment $(TR)_E$. Several improvements of this technique were indeed carried out earlier to obtain better competent cells[25-27].

Repeated cycles of heat and cold shock

The $CaCl_2$ helps DNA adsorption to the competent cell surface and the heat shock step facilitates the penetration of the adsorbed DNA into the cell cytosol. As a modification of this method, we repeatedly administered cycles of heat pulse and cold shock on

Table 2—Effect of modifications of $CaCl_2$ method on TR_E, expressed as {Number of transformants μg^{-1} of DNA} × {Final volume at recovery (ml)/Volume plated (ml)}. The data are represented as $(TR_E) \times 10^4$.

[Values represent mean ± SE of triplicate plates for each experimental set. Each experiment is repeated thrice with similar results]

Methods	pBI121	pCAMBIA1201
Protocol 1– $CaCl_2$-competent cells treated with single heat shock	1.244 ± 0.050	1.400 ± 0.060
Protocol 2– $CaCl_2$-competent cells treated with single microwave	3.196 ± 0.197	2.566 ± 0.210
Protocol 3– $CaCl_2$-competent cells treated with double heat shock	3.200 ± 0.277	3.286 ± 0.220
Protocol 4– $CaCl_2$-competent cells treated with double microwave	5.146 ± 0.120	3.936 ± 0.146
Protocol 5– $CaCl_2$-competent cells treated with triple heat shock	2.900 ± 0.080	5.186 ± 0.120
Protocol 6– $CaCl_2$-competent cells treated with single heat shock, followed by single microwave	2.590 ± 0.113	4.583 ± 0.260
Protocol 7– $CaCl_2$-competent cells pre-treated with 5% ethanol	1.800 ± 0.110	2.837 ± 0.230
Protocol 8– Ethanol-pre-treated $CaCl_2$-competent cells subjected to single heat shock followed by single microwave	2.520 ± 0.230	4.160 ± 0.260
Protocol 9– $CaCl_2$-competent cells, treated with 10% ethanol, substituting the single heat shock treatment	0.199 ± 0.010	0.238 ± 0.080

Data are found statistically significant at $P < 0.01$ (Two-way analysis of variance)

DNA adsorbed competent cells (Table 2) up to the third cycle. The second cycle of heat-cold pulses produced 2.6-folds and 2.34-folds more transformants containing pBI121 and pCAMBIA1201, respectively than the first cycle. The third cycle further increased the $(TR)_E$ for pCAMBIA1201 to a small extent, i.e., only 1.6-folds more than after the second cycle. For pBI121, a saturation in $(TR)_E$ might have occurred after the third cycle, so that it showed no further increase.

Thus, $(TR)_E$ at the second cycle was more than double of that at its first cycle and $(TR)_E$ at the third cycle was slightly greater than or almost the same as that at the second cycle, indicating a saturation effect. Thus, we performed the heat shock-cold pulse processes up to the third cycle and not beyond that. Our observation was in accordance with the earlier reports[15,21] which showed that the repeated cycles of heat and cold shocks increase the pore formation on the outer membrane, due to the release of lipid and protein molecules respectively in every cycle, concomitant with alternate rigidification and fluidization, thus favouring larger passages of DNA penetration. This probably accounted for increase in $(TR)_E$ of cells with plasmid DNA at least up to the third cycle. Excessive pore formation during the third cycle perhaps made $(TR)_E$ to attain its maximum saturation value.

Microwave treatment instead of heat shock

Recently, a very remarkable observation[17] suggested that microwave pulse, instead of heat shock increases *E. coli* $CaCl_2$ transformation. Our analysis with pCAMBIA1201 and pBI121 transformation using microwave treatment is given in Table 2. Compared to the classical single heat shock, $(TR)_E$ increased for both pBI121 and pCAMBIA1201, i.e., 2.56-folds and 1.83-folds respectively, when the DNA adsorbed competent cells were held for 1 min at 180 W in a commercial microwave oven. This improvement might be due to a more efficient heat-shock imposed by the microwave. When the competent cells were subjected to double microwave treatment with a 15 min interval on ice between treatments for cellular recovery, it further improved the $(TR)_E$, *viz*., by about 4.13-folds and 2.81-folds respectively for pBI121 and pCAMBIA1201 over the classical method with single heat shock. Thus, the microwave pulse repeated for two cycles increased $(TR)_E$ 1.5-1.6 folds over single microwave treatment, again supporting the earlier observation[17].

Combined heat shock-microwave treatment

The sequential heat shock-microwave treatment in combination with 15 min interval on ice in between augmented the overall $(TR)_E$ by about 2.1-folds and 3.3-folds respectively for pBI121 and pCAMBIA1201 with respect to single heat shocked cells. Since the reverse sequence, i.e., microwave-heat shock treatment is suggested to diminish the $(TR)_E$[17], we did not perform this analysis. It seemed that the combined treatment further potentiated $(TR)_E$ when the heat shock pulse was applied first (Table 2).

Heat shock substituted by ethanol treatment

Since the presence of 10% (v/v) ethanol in the growth medium is shown to induce the heat shock response in *E. coli*[28-30], we replaced the 42°C heat

shock step of DNA adsorbed competent cells with ethanol treatment. However, $(TR)_E$ for both pBI121 and pCAMBIA1201 decreased drastically (6.3-times and 5.9-times, respectively) i.e., approximately 6-folds with respect to control, where single heat shock treatment was subjected (Table 2).

Ethanol pre-treatment of $CaCl_2$-competent cells

As earlier stated, the more the extent of rigidification of outer membrane of *E. coli* cell, the more is its $(TR)_E$. Ethanol, an agent known to rigidify more the outer membrane of $CaCl_2$-treated competent cells, during pre-treatment produced higher $(TR)_E$[31]. Moreover, pre-treatment of $CaCl_2$-treated competent cells with 5% (v/v) ethanol for a period of 30 min caused leaching of about 40% LPS molecules from the cell surface to extracellular medium[16]. Our observation showed 2-folds and 1.45-folds enhancement in $(TR)_E$ of pCAMBIA1201 and pBI121 respectively of 5% (v/v) ethanol pre-treated competent cells, subjected to single heat shock than cells made competent without pre-treatment. Sequential heat shock-microwave treatment of ethanol pre-treated $CaCl_2$ competent cells also enhanced $(TR)_E$ of pCAMBIA1201 and pBI121 even further (3-folds and 2-folds, respectively) over $CaCl_2$-competent cells, subjected to single heat shock (Table 2).

Conclusion

The results in the present study demonstrated the $CaCl_2$ method as the best standardized transformation method and clearly indicated that very minor modifications in this classical method might have an immense potentiality in further increasing $(TR)_E$. The minor alterations like heat shock for two-three cycles, instead of one, or double microwave treatment, instead of a single 42°C heat shock can augment the $(TR)_E$ several folds. These improved protocols can thus be used as potential candidate methods of *E. coli* transformation.

Acknowledgement

Financial assistance from the Department of Biotechnology, Government of India and Bose Institute Fellowship is gratefully acknowledged. The authors thank the Director of Bose Institute, Kolkata for providing departmental facilities and especially to Mr. Jadab Ghosh for technical assistance.

References

1 Bergmans H, van Die I M & Hoekstra W P M (1981) *J Bacteriol* 146, 564-570
2 Weston A, Brown M G M, Perkins H R, Saunders J R & Humphreys G O (1981) *J Bacteriol* 145, 780-787
3 Michod R, Wojciechowski M & Hoelzer M (1988) *Genetics* 118, 31-39
4 Redfield R J (1988) *Genetics* 119, 213-221
5 Hanahan D (1983) *J Mol Biol* 166, 557-580
6 Cohen S N, Chang A C Y & Hsu L (1972) *Proc Natl Acad Sci* (USA) 69, 2110-2114
7 Mandel M & Higa A (1970) *J Mol Biol* 53, 159-162
8 Oishi M & Cosloy S D (1972) *Biochem Biophys Res Commun* 49, 1568-1572
9 Norgard M V, Kleem K & Monahan J J (1978) *Gene* 3, 279-292
10 Hanahan D, Jessee J & Bloom F R (1991) *Meth Enzymol* 204, 63-113
11 Klebe R J, Harriss J V, Sharp Z D & Douglas M G (1983) *Gene* 25, 333-341
12 Chung C T, Niemela S L & Miller R H (1989) *Proc Natl Acad Sci* (USA) 86, 2172-2175
13 Inoue H, Nojima H & Okayama H (1990) *Gene* 96, 23-28
14 *Promega Protocols and Application Guide* (1996) 3rd edn, pp. 45-46
15 Panja S, Saha S, Jana B & Basu T (2006) *J Biotechnol* 127, 14-20
16 Sarkar S, Chaudhuri S & Basu T (2002) *Curr Sci* 83, 1376-1380
17 Fregel R, Rodriguez V & Cabrera V M (2008) *Lett Appl Microbiol* 46, 498-499
18 Lee L & Gelvin S B (2008) *Plant Physiol* 146, 325-332
19 Sambrook J & Russell D W (2001) *Molecular Cloning*: *A Laboratory Manual*, 3rd edn, Vol. 1, pp. 1.32-1.34, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20 Dagert M & Ehrlich S D (1974) *Gene* 6, 23-28
21 Panja S, Aich P, Jana B & Basu T (2008) *Mol Membr Biol* 25, 411-422
22 Sarkar S, Chaudhuri S & Basu T (2002) *Indian J Biotechnol* 1, 209-211
23 Panja S, Aich P, Jana B & Basu T (2008) *Biomacromolecules* 9, 2501-2509
24 Panja S, Jana B, Aich P & Basu T (2008) *Biopolymers* 89, 606-613
25 Huff J P, Grant B J, Penning C A & Sullivan K F (1990) *Biotechniques* 9, 570-577
26 Nakata Y, Tang X & Yokoyama K K (1997) *Meth Mol Biol* 69, 129-137
27 Chen X, Guo P, Xie Z & Shen P (2001) *Antonie Van Leeuwenhoek* 80, 297-300
28 Neidhardt F C, Van Bogelen R A & Vaughn V (1984) *Annu Rev Genet* 18, 295-329
29 David B S, William A, Walter A G & Carol A G (1987) *Nature* 329, 348-351
30 Chaudhuri S, Jana B & Basu T (2006) *Cell Biol Toxicol* 22, 29-37
31 Dombek K M & Ingram L O (1984) *J Bacteriol* 157, 233-239