**Screening method discussion:**

Once the library is generated, the main issue is to identify clones with interesting properties such as higher activities or better binding capabilities (**Raymond J 2006**). This can be achieved via two fundamentally different methods- Screening- Library clones are assayed individually; or apply conditions that test all the clones simultaneously i.e. selection. The choice of the above method depends upon outcome (endpoint), experimental feasibility, and how frequently the interesting variants occur in the library (**Jackel et al., 2008; Marciano et al., 2008**). There are both cell free and cell based screening methods available for the evaluation of catalytic or binding activities and often these screening method uses model substrates possessing chromophore or fluorophore (**Leemhuis et al., 2009**). There are various screening strategies available specific for a particular experimental system e.g. chromophore based substrate screening β-galactosidase, glycosynthases or epoxide hydrolase on Agar plate (**Ben-David et al., 2008; Parikh and Matsumura 2005; van Loo et al., 2004**) or screening cytochrome P450, cyclodextrin glucanotransferase or glycosyltransferase on microtiter plate (**Kelly et al., 2008a; Kelly et al., 2008b; Bloom et al., 2006**).

Since the plasmids in our β-lactamase library have inducible promotor, and unavailability of dot blot system in the lab, we designed two step screening method in this study. In step one; we screened clones based on the expression of full length protein by SDS-PAGE. We choose this method to ensure that full length protein is being expressed which may not be the case while using dot blot method used in previous study (**Goldberg et al., 2003**). There are reports which used dot blot method for pre-screening b-lactamase library, and can be combined with selection pressure such as Ampicillin or Cefotaxime (**Marciano et al., 2008; Goldberg et al., 2003; Stemmer 1994**) to identify desired clones. In step two we screened for the activity of the enzyme, in vivo, and determined the MIC values on three different antibiotics viz Ampicillin, Cephalothin, and Ceftazidime. Those clones which expressed full length protein in step one were selected for growth assay in liquid media (Microtiter plate format) following the protocols developed earlier (**Marciano et al., 2008; Wiegand et al., 2008**). Alternate methods for determining MIC values are available such as Agar diffusion method, and Agar dilution method (**Wiegand et al., 2008; Goldberg et al., 2003**). In Agar diffusion method, the commercially available strips containing exponential gradient of antibiotics use to inhibit the lawn of bacterial colony whereas in Agar dilution method, a defined number of bacterial cells are spotted on to the LB-Agar plates containing different concentrations of antibiotics. A major drawback using Agar diffusion is that the method is limited to the antibiotic range supplied by the manufacturer and is expensive if used in high-throughput (**Wiegand et al., 2008**). Although Agar dilution method is equally efficient for growth assay, this method may become time consuming when used for screening more than one antibiotic.

We have to consider the overall methodology used in the construction of our b-lactamase library and screening design-

**First**- Given the combinatorial nature of our library, it was constructed by joining three fragments together followed by ligation with pET vector. This explains the relatively low number of potential mutant clones. In more traditional library construction approaches, mutations are generated on whole plasmid which can bypass ligation step and directly transformed in to bacterial cells. The second relatively low efficient step is the transformation. The transformation efficiency is dependent upon initial concentration of the DNA (DNA after ligation), and how efficiently the competent cells take up the DNA. If we had electroporation system, we could have achieved much greater efficiency transformation. Nonetheless, following traditional ligation and transformation protocols, we have achieved substantial efficiency at this step.

**Second**- Once we got the clones, the screening was designed to see 1) if clones are expressing desired protein, and 2) they can grow in presence of antibiotics, and determine MIC values. Since we do not have equipment to carry out dot blot screening, I decided to screen clones using SDS-PAGE. This method is much more stringent than dot blot and eliminate any and all defective clones e.g. out of 100 clones screened by this method, I got about 40-60 good clones. These clones then used in the growth assay, initially, I tried to use Agar dilution method (**Goldberg et al 2003**), but it did not work, partially due to inefficient IPTG induction in Agar plate. Other reports in the literature which used Agar plate method; their library was constructed in constitutively expressing TEM-1 promoter. This was significant point which drove me to find alternative microtiter plate method published earlier (**Marciano et al., 2008; Wiegand et al., 2008**). It is matter of speculation that if we had TEM-1 promoter, we might have gotten higher percentage of “good” clones because expression is not dependent upon IPTG.

**Third**- The screening methods discussed for some of the enzymes earlier (β-galactosidase, glycosynthases or epoxide hydrolase, cytochrome 450 etc) is **non comparable** to the b-lactamase screening. Those enzymes were grown in the bacteria and then processed to evaluate the activities of the respective enzymes. In case of b-lactamase, we can screen clones based on the basis of their growth in presence of antibiotics. If we have to compare those screening efficiencies with b-lactamase, we might have to introduce chromophore based substrate for screening and extra step in the protocol i.e. lysis of the bacteria, clarify the supernatant then add substrate for activity assay in 96 well format. The main problem with the chromophore based substrate (e.g. **Nitrocefin**) is that they do not represent an ideal substrate (**Leemhuis et al., 2009**). There is another problem with single substrate model based activity; b-lactamase exhibit varying degree of activities on different substrate e.g. a particular b-lactamase mutant might have entirely different kinetic activity on different substrates (varying substrate specificity; this is why researcher use more than one substrate during screening/activity/kinetic assay).