Colony PCR

We use a colony polymerase chain reaction as a screening test to determine whether the transformed bacterial colonies on agar plates contain the plasmid with the integrated insert. Under optimal conditions a spanning region between vector backbone and of the insert are amplified in this PCR reaction and become visible as a specific band during the following agarose gel electrophoresis. They prove that both key steps of the cloning procedure took place, that means DNA integration into the vector and transformation of bacteria with the altered vector. If the cloning process failed, however, no specific amplification occurs and consequently no or an unspecific band appear during the gel electrophoresis.

Colony PCR for cloning strategy (I) using TA cloning

Usually, colony PCR testing would not be necessary in this case, because a common blue-white-screen would be sufficient. Successful integration of foreign DNA into the TA vector plasmid normally causes a *lacZalpha* knock out, and transformed bacterial colonies will appear white on a selection plate containing X-gal. In contrast, colonies that do not possess the insert, will appear blue, because their *lacZ* gene is not interrupted. In our cloning experiments, however, we use DNA inserts which themselves all have a functional *lacZ* gene in them. This means, all colonies may appear blue eventually, regardless if they contain the insert or not. That is why we deserted to using colony PCR.

Colony PCR for cloning strategy (II) using restriction enzyme sites and ligation

Our toehold switch sensors contain a lac Z reporter enzyme gene, we may be able to do a blue-white screening with bacterial colonies in this strategy, but the toehold design may prevent active LacZ expression without the presence of its specific target RNA. Since we could not predict sensor leakage in E. coli colonies, we decided to execute colony PCR for identification of bacterial clones with the correct insert.

A colony-PCR is a variant of PCR which is used to directly amplify specific regions of vector DNA from bacteria without having to extract and clean the DNA beforehand. Instead of adding a clean template DNA strand to the PCR reaction mix, miniscule amounts of whole bacteria from the colonies in question are used. First, colonies are picked with a sterile pipette tip and transferred into purified water, where they get osmotically lysed. Then, the PCR master mix is added to the bacteria lysate and PCR is performed in a thermocycler.

In all our cloning experiments, the DNA inserts contain the same *lacZ* gene with the same T7 promotor in front. In principle, this allows us to amplify the same sequence in all colony PCRs.

Choice of primers:

- Depending on the specific step we use different forward primers. After cloning our sequences into the TAeasy vector we use the forward primer ’XbaLT7prom(+6).FOR’ [also referred to as ‘T7+6’] that binds to parts of the T7 promoter. However, after cloning our sequences into the pSB1C3 iGEM shipping vector, we use the iGEM standard forward primer ‘VF2’ which is complementary to the backbone. Resulting PCR products then contain both: backbone DNA and inserted DNA. This design decreases the chances of false positive test results.
- As reverse primer we either use ‘Seq Primer 1’ (also referred to as ‘Seq1’) or ‘Seq Primer 2’ (also referred to as ‘Seq2’). Both of them bind within the insert *lacZ* of our toehold switches. Our full length *lacZ* gene differs from the vector *lacZ alpha* gene, that is a shorter gene isoform and part of TA vector backbone, to which the reverse primers do not bind. The two reverse primers do not differ a lot in their function and they can be interchanged in principle, however, we tend to prefer ‘Seq2’ due to clearer bands in agarose gel electrophoresis.

Protocol

Reagents and Materials

- Promega GoTaq® Flexi DNA Polymerase
- Promega 5X Green GoTaq® Flexi Buffer
- 10 mM dNTPs
- 50 mM $MgCl_2$
- Nuclease free water
- Specific Primers
    - forward primers:
        - ‘XbaI.T7prom(+6).FOR’ [‘T7+6‘]: GCCGCTTCTAGAGCGAATTAATACGACTCAC
        - ‘VF2’: TGCCACCTGACGTCTAAGAA
    - reverse primers:
        - ‘Seq Primer 1’ [‘Seq1’]: CAAAGCGCCATTCGCCATTCA
        - ’Seq Primer 2’ [‘Seq2’]: GATAGGTCACGTTGGTGTAGATG
- Thermocycler: ‘Mastercycler Gradient‘ by Eppendorf

Procedure

- Pipette PCR mastermix:

Table 1: Mastermix pipetting scheme

Component	**Volume (µl) for 25 µl reaction**
5X Green GoTaq® Flexi Buffer (Promega)	5
10 mM dNTPs	0.5
10 µM forward primer*	0.5

10 µM reverse primer**	0.5
50 mM $MgCl_2$	1
Nuclease-free water	7.375
total	25

*forward primer: either ‘T7+6‘ or ‘VF2’
**reverse primer: either ‘Seq1’ or ’Seq2’

- Add 10 µl of purified water to PCR tubes
- Pick colony with tip of pipette. Transfer picked colony into the PCR tubes and pipette up and down numerous times to lyse the bacteria.
- Add 0.125 µl of GoTaq® Flexi DNA Polymerase per PCR reaction to the PCR mastermix and vortex.
- Transfer 15 µl of PCR mastermix to each PCR tube containing 10 µl of bacteria lysate.
- Run PCR using the following *slowdown* PCR program:
    - Initial denaturation: 3 min 95°C
    - *Slowdown* cycles:
        - Denaturation: 30 sec 98°C
        - Annealing: 30 sec; first cycle 70°C; then -0.5°C/cycle
            - if using forward primer ‘T7+6’, repeat till annealing temperature reaches 60°C
            - if using forward primer ‘VF2’, repeat till annealing temperature reaches 56°C
        - Extention: 40 sec 72°C
    - Following the slowdown part of this PCR, run **25 standard PCR** cycles:
        - Denaturation: 30 sec 98°C
        - Annealing: 30 sec 60°C / 56°C respectively
        - Extention: 40 sec 72°C
    - Final extention: 5 min 72°C
    - Cool at 4 °C

- Prepare a 1% Agarose Gel and load 3 µl of PCR product onto the gel (loading buffer already included in the polymerase buffer). Perform electrophoresis at 100 V for approximately 1 hour.