Insert Screening Protocols for NEB PCR Cloning Kit

Overview

Screening for inserts can be performed by colony PCR, restriction enzyme digestion or PCR of mini-prep plasmid DNA, or by sequencing of the mini-prep plasmid DNA.

Many DNA polymerases are suitable for colony PCR. For inserts < 1 kb, NEB recommends One*Taq* DNA Polymerase (NEB #M0480) or One*Taq* Hot Start DNA Polymerase (NEB #M0481). *Taq* DNA Polymerase (NEB #M0267) can also be used. For inserts > 1 kb NEB recommends One*Taq* formulations, LongAmp T*aq* DNA Polymerase (NEB #M0323) or LongAmp Hot Start *Taq* DNA Polymerase (NEB #M0534); the LongAmp *Taq* formulations are especially useful for larger inserts. These products are also available in easy-to-use master mix formats. Following your choice of DNA polymerase, use the provided sequence of the cloning analysis forward and reverse primers and the Tm calculator found on the NEB website (TMCalculator.neb.com) to determine the annealing temperature for your PCR reactions.

Screening Protocol 1: Colony PCR using One*Taq* 2X Master Mix with Standard Buffer (NEB #M0482) or LongAmp *Taq* 2X Master Mix (NEB #M0287)

1. Prepare a PCR mix of sufficient volume to allow 50 µl per screened colony:

	AMOUNT	FINAL CONC.
One*Taq* or LongAmp *Taq* 2X Master Mix	25.0 µl	1 X
Cloning Analysis Forward Primer (100 µM)	0.15 µl	300 nM
Cloning Analysis Reverse Primer (100 µM)	0.15 µl	300 nM
H_2O	24.7 µl	
Total	to 50 µl	

2. Use a sterile toothpick or pipette tip to pick an individual colony, and dip into each amplification reaction. To create a stock of each individual colony, either dip the same toothpick or pipette tip into 3 ml growth media containing ampicillin, or use a separate agarose-ampicillin plate to prepare a streak or patch of the colony material.
   2a. Transfer the reactions to a thermocycler and perform PCR with the following conditions:

STEP	TEMP	TIME
Initial Denaturation	94°C	2 minutes
30 Cycles	94°C	15 seconds
	53–57°C*	15 seconds
	68°C	60 seconds/kb
Final Extension	68°C	5 minutes
Hold	4–10°C	∞

**For OneTaq, use 53°C; for LongAmp, use 57°C*

3. Load 5 µl of each completed PCR onto an agarose gel alongside an appropriate DNA ladder [e.g., Quick-Load Purple 1 kb Plus DNA Ladder (NEB #N0550)]. For reference, the amplicon length in the absence of an insertion would be 309 bp in length. The amplicon length with the positive control insert would be 1312 bp.

Screening Protocol 2: Sequence Analysis

The Cloning Analysis Forward and Reverse Primers can also be used for sequencing inserts. This can be performed with purified plasmids from overnight cultures from each colony, or with amplicons from the above colony screening PCRs. The primers anneal 155 bp upstream and 154 bp downstream (measured from the 5´ end of each primer to cloning insertion site), ensuring complete reads of the insert.