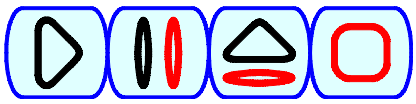
pCURE™2 kit

for the elimination of F-like plasmids from Enteric Bacteria (Enterobacteriaceae)



Patent pending

Instruction manual version 1.0

This kit uses a plasmid, pCURE™2, which interferes with the replication and population maintenance functions of F-like plasmids, in *Escherichia coli* and other bacteria of the Enterobacteriaceae. By interfering with both F-plasmid replication and post-segregational killing systems, the kit offers a way of eliminating these plasmids without stressing the host bacterium.

The plasmid pCURE™2 carries:

Region	**Purpose**
kanamycin resistance gene	selection
penicillin resistance gene	selection
IncP oriT	mobilisation
sacB	counterselection
incC	interferes with incFIA replicon
repFIB	interferes with incFIB replicon
copA/copB	interferes with incFIIA replicon
copA/copB	interferes with divergent incFIIA replicon
sok	prevents host killing
pemI	prevents host killing
letA	prevents host killing
flmC	prevents host killing

This kit contains the following components:

Instruction booklet

DNA

Store at 4ºC

pCURE™2	displacement plasmid (20 µl of 50 ng μl^{-1})
pAKE604	negative control for displacement (20 µl of 50 ng μl^{-1})

Diagnostics

Store at -20ºC

PCR primer solutions (750 µl solutions of 5 pmol μl^{-1} of each primer):

pcrVector	This solution contains a pair of primers, specifically recognising the pCURE™2/pAKE604 replicon and adjacent backbone.
pcrRepFI	This solution contains four primers, recognising regions in repFIA and repFIB replicons.
pcrRepFIIA	This solution contains five primers, recognising a region of repFIIA in four different sub-sequence families.

Disclaimer: For research use only. Use safety precautions appropriate to your bacterial strains.

Overview of pCURE™2 kit use

3,4 Transform selected strains with pCURE2 and pAKE604

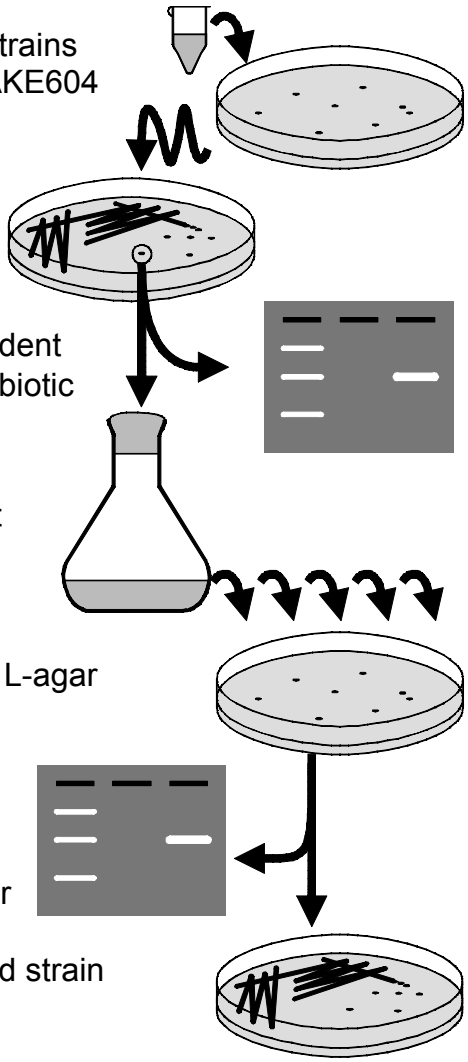
5 Purify to single colonies

6 Check for loss of resident plasmid by PCR, antibiotic sensitivity or plasmid isolation

7 Grow in broth without selection

8 Dilute and spread on L-agar containing sucrose

9 Check for loss of pCURE2 by PCR, antibiotic sensitivity or plasmid isolation, and preserve modified strain



Use of pCURE™2 kit

1) Check strain has an F-like plasmid

Before attempting to eliminate a plasmid, first check that it is likely to be a target for pCURE™2. If you already know that the plasmid you wish to eliminate is F-like, you can skip this step, although you will still need a test to confirm plasmid loss.

We provide primer solutions which will detect the F-like replicons (pcrRepFI and pcrRepFIIA). Use these in the PCR protocol in Appendix A to test your strain. A product of approximately 0.5 kb in one or both of these reactions indicates the presence of an F-like plasmid. Larger products are possible, and may also indicate an F-like plasmid.

If neither PCR reaction yields a product, it is still possible that the strain carries an F-like plasmid which can be eliminated by pCURE™2. The plasmid sequence may be divergent in the region of one of the PCR primers supplied. If you believe your strain may carry such a divergent F-like plasmid, you could try the PCRs with reduced annealing temperatures (although this can lead to cross-reaction against pCURE™2), or use your own test during or after the procedure to test for loss of the plasmid.

Your strain carrying an F-like plasmid will be called the 'F-strain' in this protocol.

While throughout this protocol we suggest you test for the presence of the F-like plasmid using PCR, you may prefer to determine its presence in another manner. Any phenotypic trait known to be encoded on the plasmid may be detected, or plasmid DNA may be isolated and identified (although in the latter case the plasmid will not be detected if it has integrated into the bacterial chromosome).

2) Preparation for curing procedure

It is necessary for your F-strain to be susceptible to kanamycin, penicillin or both. You should know or determine this before proceeding. Suitable concentrations for selection in *E. coli* are usually 50 μg ml^{-1} kanamycin for broth and agar, 150 μg ml^{-1} for penicillin G (benzylpenicillin) in broth and 300 μg ml^{-1} in agar, or alternatively 100 μg ml^{-1} ampicillin for broth and agar. Henceforth we shall refer to 'antibiotic containing', or just 'antibiotic' media.

To confirm that your transformation is working as expected, we recommend that you perform a positive control, at least the first time you use this method. If you wish to do this, use an *E. coli* control recipient – a strain which you know to be transformable – in parallel with the F-strain for this procedure.

- If you wish to transform your strain using the recommended protocol, put 100 ml of sterile 0.1M $CaCl_2$ solution at 4ºC.
- Set up an overnight culture of your F-strain and control recipient *E. coli*.
- Prepare a minimum of 16 L-agar plates with antibiotic.
- You will also need at least four L-agar plates supplemented with 5% sucrose, and three with 5% sucrose and antibiotic. These are not required until steps 8 & 5 respectively.
- To prepare 5% sucrose agar, if you have pre-prepared bottles of L-agar, it is sufficient to add 10 ml 55% pre-warmed (at least 48ºC) sterile sucrose solution per 100 ml molten L-agar.
- Finally, you will need a plain, non-selective L-agar plate on to which to streak your derived strain (or use a spare 5% sucrose plate).

3) Make recipient strain competent

If you have a good, working method for making your strain competent, use it. (If you wish to electroporate your cells you will need to purify the vector DNA supplied; it is stored in $^1/_{10}$ TNE.) Otherwise, use the following:

- Inoculate 50 ml of L-broth with 0.5 ml of an overnight culture of your *E. coli* strain.
- Grow at 37ºC in shaken culture to an optical density at 600 nm (OD_{600}) of between 0.3 and 0.5 (this generally takes about 2 hours).
- After this step keep the bacteria on ice and ensure all solutions used are ice cold.
- Transfer batches of 5 to 10 ml of this culture to sterile centrifuge tubes and pellet by spinning in a bench centrifuge at around 5000 g and 4ºC for 5 min.
- Pour off the supernatant and re-suspend the cells in 2 ml of ice cold 0.1 M $CaCl_2$ solution. After storage on ice for 20 minutes, re-centrifuge the tubes and remove the supernatant. During this incubation the cells become increasingly fragile. Do **not** vortex them after this step.
- Resuspend the cells in 0.5 ml $CaCl_2$ solution by **gentle** flicking of the tube. After a further 20 min on ice, the bacteria are competent.
- Tubes can be stored at 4°C for up to 2 weeks.

4) Transform competent cells with pCURE™2 (and separately pAKE604)

We very strongly recommend that you perform three tests: transform with pCURE™2; transform with a negative elimination control (pAKE604); and also a negative transformation control using no vector DNA. If you are also transforming control *E. coli*, you will have six samples:

plasmid recipient	pCURE™2 F-strain	pAKE604 F-strain	no DNA F-strain
plasmid recipient	pCURE™2 ctrl recipient	pAKE604 ctrl recipient	no DNA ctrl recipient

If you have your own transformation protocol use that, otherwise use the following:

- Ensure that the competent cells are evenly suspended by gentle flicking – they tend to settle in storage.
- Into a cold microfuge tube, mix a 100 µl aliquot of your competent cells with 1 µl of vector DNA by gentle tapping.
- Store on ice for 60 minutes.
- Heatshock the cells by incubating at 42ºC in a waterbath for 90 seconds.
- Return tubes to ice for 1–5 minutes.
- Add 500 µl of L-broth and mix by inverting several times.
- Incubate at 37ºC for 1 hour. (You may wish to put on a shaker after a few minutes of incubation, to improve growth.)
- Plate 100 and 400 µl aliquots of the transformation mix on L-agar with antibiotic. This selects for the incoming plasmid. (Your plates must be quite dry to receive 400 µl of liquid. Alternatively pellet cells and resuspend in 100 µl.)
- Prepare L-agar sucrose+antibiotic plates for the next stage, if you have not already done so.
- Incubate your plates at 37ºC overnight.
- You should have colonies on the four transformations with added plasmid DNA, and no colonies on the no-DNA controls.

5) Restreak colonies to create F-free clones

- For your F-strain pCURE™2 transformations, pick three or more representative colonies. Also choose one or two F-strain pAKE604 transformation colonies for use as a positive control in the following PCR reactions.
- For each chosen transformation colony, streak to single colonies on L-agar containing antibiotic. Also streak out the pCURE™2 transformation colonies on to L-agar containing sucrose+antibiotic.
- Incubate these plates at 37ºC overnight.
- If any of your pCURE™2 restreaks on sucrose+antibiotic plates have grown beyond the initial streak, discard those clones (genetic integrity of the *sacB* gene on the plasmid is slightly unstable, and its loss prevents counter-selection).

6) Confirm loss of F-like plasmid

- Choose a well-separated colony from each plate. Test these by PCR, or another method of your choice. The pCURE™2 clones which have successfully lost the F plasmid will be used to inoculate further cultures. Therefore either you must be able to return to the picked colony, or patch on to another plate to use later.
- Perform PCR using the protocol in Appendix A with primer pairs specific for F plasmid. Both PCRs should be negative for your pCURE™2 transformants, but one or both should give bands in the pAKE604 control transformation as for your parental strain.
- It is recommended that you also perform PCR using primers specific to the pCURE™2/pAKE vector. This primer pair (pcrVector) should give a product in your transformed strains, but not in your parental strain.

7) Grow without selection to allow pCURE™2 loss

The pCURE™2 plasmid is slightly unstable, being lost from a daughter cell at a proportion of cell divisions. A few generations without selection are required to enable cells losing the plasmid to survive.

- Pick one or more colonies you have determined to lack the F-like plasmid and inoculate non-selective L-broth cultures.
- Grow the cultures overnight at 37ºC with shaking.
- Prepare L-agar plates supplemented with 5% sucrose for the next stage, if you have not already done so.

8) Select against bacteria carrying pCURE™2

Cells carrying pCURE™2 should not grow on media containing sucrose, as pCURE™2 encodes SacB, which confers sucrose sensitivity on Gram-negative bacteria.

- Set up a series of five 10-fold serial dilutions from your overnight cultures. We use 0.9 ml sterile distilled water plus 100 µl cell suspension.
- Spread 200 µl of 10^{-3}, 10^{-4} and 10^{-5} diluted culture on to 5% sucrose L-agar plates.
- Incubate these plates overnight at 37ºC.
- Pick one or more representative separated colonies and streak to single colonies on non-selective agar. You can check that these clones are pCURE™2-free by also making a test patch on antibiotic L-agar.
- Incubate these plates overnight at 37ºC.

9) Verify strains

- Confirm that your resultant strain is pCURE™2 free using the primer pair pcrVector. Use a sample from your transformed strain as a positive control.

Appendix A: PCR protocol and associated steps

To generate PCR template from a colony, first prepare 'boiled colony template':

- Use a small sterile implement such as a 200 µl pipette tip to pick up a portion of a colony. The sample taken should be visible, but larger samples are undesirable.
- Resuspend the sample in 100 µl of sterile distilled water in a microfuge tube.
- Incubate this suspension in a boiling water bath for 10 minutes, then remove and allow to cool.
- Pellet the cellular debris using a microfuge for 2 minutes at maximum speed.
- The supernatant can be used as template solution in a PCR reaction.
- Store the tube on ice for immediate use, or store at -20ºC for later use (you may need to re-pellet the cellular debris).

The success of PCR reactions varies based on many factors including the make of polymerase enzyme, the thermal cycler used, the quality of purified water and many more. Therefore we recommend using positive and/or negative controls as appropriate wherever possible.

While we have endeavoured to give robust conditions, these instructions should therefore be seen as an initial guide, rather than an optimised protocol for your system.

As these are merely diagnostic PCRs the error rate is not important, therefore a cheap form of DNA polymerase enzyme such as *Taq* is sufficient.

The primers are stored in a protective buffer which adds 0.1 mM EDTA to the reaction. Therefore you should add extra magnesium ions to account for this. We use a total of 1.1 µl of 50 mM $MgCl_2$ solution per reaction.

Reactions can be set up as follows:

4 µl boiled colony template
5 µl PCR primer pair solution (=25 pmol of each primer)
4 µl 2.5mM each nucleotide dNTP stock (or as recommended by polymerase manufacturer)
x µl polymerase buffer (as recommended by polymerase manufacturer)
x µl $MgCl_2$ solution (if not included in the above buffer)
x µl polymerase enzyme (as recommended by polymerase manufacturer)
x µl water, to make it up to 50 µl.

A default thermal cycling protocol is:

Temp (ºC)	Time (sec)	Description
95	120	initial denaturation
10 cycles of:		
59	30	primer annealing
72	35	extension
94	30	dissociation

followed by 15 cycles in which the extension step is increased by 5 seconds per cycle,

and a final stage to extend partial products

59	45	annealing
72	600	final extension

Confirm the products by running 10 µl by gel electrophoresis as you do normally.
See the next page for sample reactions

Sample diagnostic PCR reactions

The plasmids pCURE™2 and pAKE604 were used to transform three F-strains. Three transformant colonies with pCURE™2 and one with pAKE604 were streaked to single colonies on kanamycin plates. Individual colonies were used to create boiled colony template for the following reactions. The PCR reactions shown are generally equivalent to those performed at step 5 of the protocol.

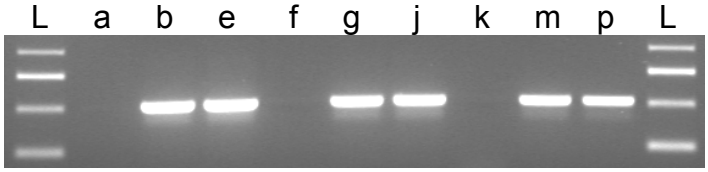
Lane labelling scheme

In the following gels, each lane is assigned a letter identifying the template material.
Not all templates are used in each gel.

strain	EMG2	unclassified	DH5α
plasmid	F1-1	F-like	pKDSC50
Parental strain	a	f	k
pCURE™2 c.1	b	g	m
pCURE™2 c.2	c	h	n
pCURE™2 c.3	d	i	o
pAKE604	e	j	p

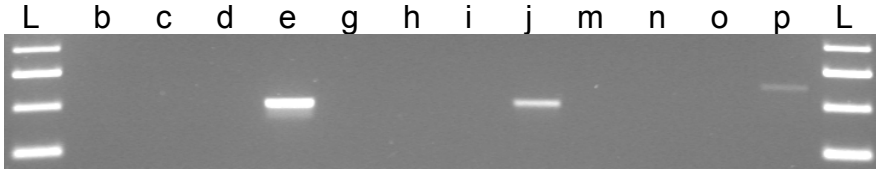
L DNA ladder. Visible bands are 1000, 750, 500 and 250 bp.

pcrVector primers



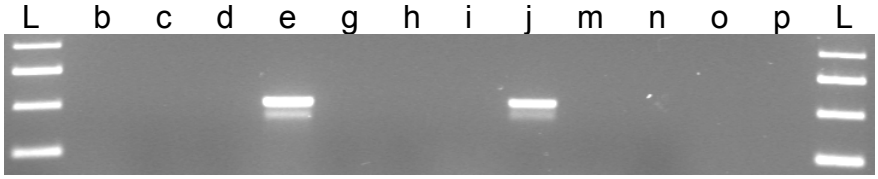
Only a single pCURE™2-carrying clone is shown for each strain. Lanes **b**, **e**, **g**, **j**, **m** and **p** have bands due to the presence of the pCURE™2 or pAKE604 plasmid. The parental strains in lanes **a**, **f** and **k** do not contain these plasmids so are free of bands.

pcrRepFIIA primers



The negative control plasmid pAKE604 does not eliminate F-like plasmids, therefore PCR products identifying the IncFIIA replicon are visible in lanes **e**, **j** and **p**. These bands are equivalent to those seen in the parental strains.
The presence of pCURE™2 in the samples in the other lanes has led to the elimination of the F-like plasmid, and hence the absence of any bands.

pcrRepFI primers



Bands are visible in lanes **e** and **j**, because the negative control plasmid pAKE604 has not eliminated the F-like plasmid. Two bands are visible as these F-like plasmids carry both repFIA and repFIB replicons. No bands are visible in lane **p**, because although the F-like plasmid present carries an IncFIB replicon, the sequence is divergent at the binding site of one of the PCR primers.
The presence of pCURE™2 in the clones in the lanes **b**, **c**, **d**, **g**, **h** and **i** has led to the elimination of the F-like plasmid, and hence the absence of any bands.