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Dirty Plasmid Used As A Quick And Low-Cost Method to Identify Bacterial Colonies With A Recombinant Plasmid

KEYWORDS

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ABSTRACT We reported a "dirty plasmid method" in which phenol-chloroform is used to break bacterial cells to release plasmid that appears as one single conformation, thus allowing researchers to distinguish the recombinant plasmid by its slower migration in agarose gel from the faster migrating empty vector. This procedure does not involve RNase and other chemicals or buffers and, together with a "dirty PCR method" that uses polymerase chain reactions (PCR) to determine the insert's orientation, makes it quick and low in cost to screen a large number of bacterial colonies for identification of the recombinant plasmid with the correctly-oriented insert.

Molecular cloning requires distinguishing those bacterial colonies that bear the recombinant plasmid from those bearing the empty plasmid vector. In 1994 Akada described a quick procedure for screening the colonies [1], and later others reported similar methods [2, 5, 6]. Because the DNA insert increases the size, i.e. the molecular weight, of the plasmid, in these earlier methods the recombinant plasmid can be distinguished from the empty vector by its slower migration in agraose gel, although the difference in the gel is small because plasmids are circular DNA. After nearly two decades, many new vectors have emerged with much larger sizes that shrink the difference from the insert-containing counterpart in gel and make it unclear whether the previous methods are still useful. Moreover, the previous methods cannot determine the orientation of the insert in the vector, whereas expression of the insert-encoded protein requires the correct orientation. In today's molecular cloning, a DNA fragment is often cloned into a cloning vector first, and the resultant bacterial colonies can be dichotomized easily by their colors and thus do not need to be screened with a previous method. After propagated in the bacteria, the DNA insert is excised out from the cloning vector and purified from agarose gel using such as our simple method [9], and then is cloned again into an expression vector, followed by identification of a desired bacterial colony via a blind and random screening. Sometimes only a wee portion of the colonies are the desired ones, making the screening of a large number of colonies inevitable.

Because each of the earlier methods has its strength and weakness, we established our own procedure by combining and modifying the earlier ones. In our method, after having decided how many colonies should be screened, the cloner should prepare Eppendorf (EP) tubes accordingly by adding 300-400 µl of Luria-Bertani broth (LB) into each tube. A toothpick or pipette tip is used to pick up each bacterial colony from the agar dish and to inoculate the bacteria into the LB (Fig 1A). The tubes are then capped and cultured with shaking. After an overnight culture, 50 µl of the bacteria, 80 µl of a phenol-chloroform (1:1) mixture, and 10 µl of 6x DNA gel loading dye (used for loading DNA into agarose gel) are added into each of a new set of smaller (500-µl) tubes, followed by vortexing at the highest speed for 60 seconds to break the bacteria. After centrifugation in a mini-centrifuge for 2 minutes, 30 µl of the aqueous supernatant is transferred into a thick 0.8-1% agarose gel that contains ethidium bromide (EB), followed by electrophoresis and photographing the gel, as routine.

In the gel, all plasmids will migrate much faster than the bacterial DNA but much slower than the 23S, 16S and 5S ribosomal RNAs (rRNA) [3, 7] (Fig 1B). The crude (dirty) plasmid only has one conformation, unlike the plasmid isolated from many other methods that has several, such as relaxed and supercoiled, conformations and migrates as multiple bands in agarose gel, which makes it possible to compare the insert-containing plasmid with its empty vector. Although still in its circular form, the plasmid with an insert migrates slower than its empty vector, but the distance between the two in the gel is much smaller than the length of the insert, and thus it has little use to include linear DNA markers of molecular weight as references for circular plasmid. Sometimes a plasmid may contain multiple copies of the DNA fragment and migrates even slower (late 9 in figure 1B). Figure 1C presents actual experimental results showing that in three different pairs of empty vector and recombinant plasmid, all plasmids with an insert ran slower than their empty vector counterparts. In our practice, this dirty plasmid method has been good to detect an insert as short as 450 base pairs (bps) in the pcDNA3.1 vector, but we have not yet tried shorter inserts. Once a positive clone is selected, the remaining LB in the

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corresponding EP tube will be used for a larger-volume culture to obtain more plasmid.

If the plasmid cannot be discerned in the gel, in a repeat the cloner can 1) collect and use more bacteria by centrifuging the bacteria and then aspirating, from the bottom of the tube, concentrated bacteria, 2) vortex the bacteria harder to ensure the bacterial breakage, and 3) make a thicker gel with deeper wells to accommodate more plasmid. If the cloner wants to cut the cost at the expense of time, less (100 µl) LB and smaller (500-µl) tubes can be used with a longer large-volume culture later. A 96-well plate can also be used to replace many tubes, as described by Davis et al [2], if an equipment for centrifuging the plate is available.

If the insert's orientation needs to be determined, polymerase chain reactions (PCR) can be used in a second screening of several already-selected colonies with bacteria as dirty templates to produce dirty PCR product [4, 8]. In this "dirty PCR method", which can also be used to replace the above procedure as the initial screening, a 96-well plate or a set of small (500-µl) EP tubes will be prepared by adding 100 µl of LB into each well or each tube, besides a new agar dish marked at its bottom (Fig 2A). In addition, PCR tubes should be set up by adding all PCR reagents except the template, to a final volume of only 10-20 µl to save the reagent. Each bacterial colony picked up with a toothpick or a pipette tip will be 1) first inoculated onto the new agar dish, 2) then rinsed in the LB, and 3) lastly rinsed in the PCR tube (Fig 2A). These steps make the bacteria "contaminate" the new agar dish, the LB and the PCR reagents. The forward (F) PCR primer should be located at the vector and the upstream of the insert, and usually is T3, T7 or M13, while the reverse primer should be located in the insert at an asymmetric position, much closer to either the 5' (R1) or the 3' (R2) end of the insert (Fig 2B). The correct PCR product should be a DNA fragment obviously smaller (when using the F/R1 pair) or larger (when using the F/R2 pair) than half of the insert's size, whereas the opposite result indicates the wrong orientation. Because the bacteria are broken down only after a few thermal cycles, the PCR should be carried out for 40-45 cycles. While the PCR is going on, the EP tubes or the 96-well plate and the new agar dish should be cultured in proper incubators, with the EP tubes being shaken.

Sometimes many bacteria remain intact during PCR. In a repeat, one additional 96-well plate containing 30 µl of detergent (such as Tween 20 solution) and another 96-well plate containing deionized water, in each well, should be set up. After it has been rinsed in the LB, the toothpick or tip with bacteria is dipped into the detergent a few times to make the bacteria fragile and then dipped into the water to wash away the detergent, before it is rinsed in the PCR reagents.

Once the PCR results in an anticipated band, visualized by EB in agarose gel, the LB in the corresponding well or tube will be used for a larger-volume culture. Sometimes the bacteria do not grow in the large-volume culture, as often experienced with some earlier methods. Inoculating the bacteria in both an agar dish and a liquid medium allows the cloner to save time when the bacteria in the plate or tube are good enough, while otherwise still having the colony in the agar dish as a backup.

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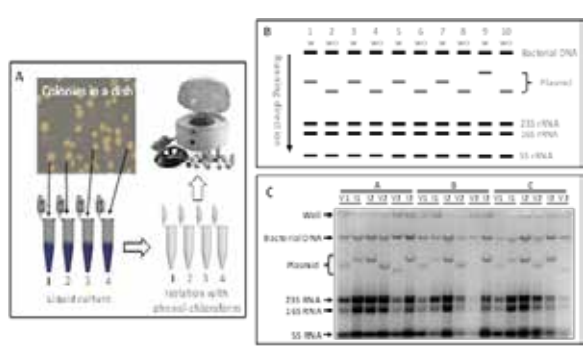
Legends to figures**:**
Fig 1: Illustration of dirty plasmid method. A: Colonies selected from the agar dish are picked up and inoculated into EP tubes for culture. An aliquot (50 µl) of the bacteria is then transferred to smaller tubes for isolation of plasmid with phenol-chloroform, followed by a quick centrifugation in a mini-centrifuge. B: About 30 µl of plasmid-containing aqueous supernatant is fractioned using electrophoresis in EB-containing agarose gel. As illustrated, all plasmids should migrate much faster than the bacterial DNA but much slower than the ribosomal RNAs (rRNA). The plasmid with (W) an insert always migrates slower than its counterpart without (WO) an insert. If multiple copies of the DNA fragment are inserted, which occurs occasionally, the plasmid migrates even slower, as illustrated in lane 9. C: In an actual experiment, the expression vectors pcDNA3.1 (V1), pcDNA3.0 (V2) and pEGFPC1 (V3) were inserted with a DNA fragment of 2500 (I1), 2600 (I2) or 2700 (I3) bps, respectively. The success of the insertion was verified elsewhere. The "dirty plasmid" preparation was repeated three (A, B and C) times. The dirty plasmids were loaded into a 1% agarose gel blindly with their identification disclosed later. Note that in each pair, the empty vector migrates faster than its insert-containing counterpart.

Fig 2: Illustration of dirty PCR method. A: Bacterial colonies picked from the original agar dish are inoculated onto a new agar dish that was pre-marked at the bottom, and then transferred, by rinsing, into the corresponding LB-containing small EP tubes and lastly into the corresponding PCR tubes already containing all PCR reagents except the template. If a PCR result indicates a desired insert with the correct orientation, the corresponding colony in the EP tube should be used for a large-volume culture. If the bacteria do not grow in the large culture, the same colony in the new agar dish as the backup should be used. A 96-well plate (not shown) can be used to replace many EP tubes, but without shaking the bacteria do not grow as robustly as in the tube, thus making the post-screening time longer. B: Illustration of asymmetric primers for determination of the orientation of the insert (the black thick dash line with its arrow indicating the 5'-to-3' orientation) in the vector (blue curve). A forward primer (F) located at the vector and the upstream of the insert, paired with a reverse primer (R1) close to the 5' end of the insert in PCR, should yield a DNA fragment shorter than half of the insert's size. On the other hand, the same forward primer paired with a reverse primer (R2) close to the 3' end of the insert should yield a DNA fragment larger than half of the insert's size. The opposite PCR result indicates the wrong orientation.



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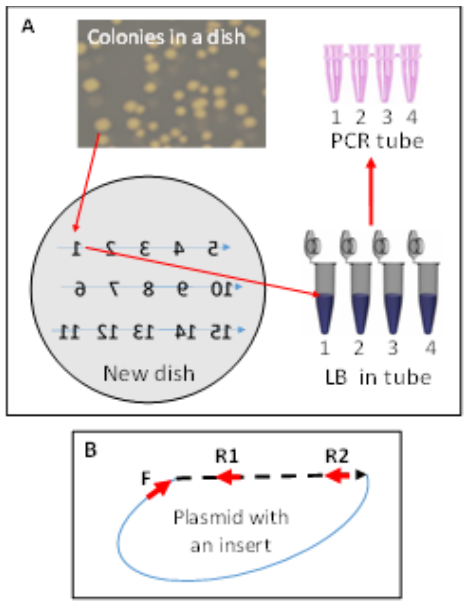

Fig. 2.

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