

Protocol for restriction digestion of plasmid & insert, purification, and ligation

NOTES: First quantify the plasmid (ideally by gel comparison, not nanodrop), and quantify the insert DNA (usually a column-purified PCR product; nanodrop is OK for this) then set up digests, as below. A typical setup would be 100 ng plasmid and 100 ng insert in a digest volume of 100 μ l.

Check which restriction buffer is appropriate. For double digests, it's OK to use a buffer which gives 100% activity for one enzyme, and 75% activity of the other, but lower than this is not good. Also check the appropriate digest temperature, its usually 37°C, but not always.

<< note – the plasmid and insert digests are described separately and sequentially below for clarity, but these can and should be set up at the same time >>

DIGEST OF PLASMID

1. Calculate how much volume of plasmid you need to use to get 100 ng – call this 'X', and calculate how much water to add to the digest; this is (100 – 10 – 1 – X) – call this 'Y'.
2. Retrieve 10 x restriction buffer from freezer, thaw completely, and vortex to mix. The same tube of buffer can be used many times, if you are careful with your aseptic technique.
3. Retrieve the plasmid from the freezer, allow to thaw, (e.g. in 37°C waterbath, or rub in your hands, or on bench etc), then put it on ice when it is thawed. Its not good to leave the plasmid stock at room temp or above for prolonged periods or it may degrade due to traces of nucleases.
4. Retrieve the restriction enzyme(s) from the freezer, put IMMEDIATELY on ice. These are heat-sensitive and you need to look after them. Do not leave them at room temp. Keep on ice while setting up the reaction, then immediately put back in freezer. These don't need to be thawed, they are in a glycerol solution which doesn't freeze at -20°C.
5. Label your tube(s), then set up the digest(s) by adding the ingredients in the following order. Make sure you use excellent aseptic technique, and change tips every time. Its OK to set this up at room temp, the reaction tube doesn't have to be on ice.

- 10 μ l of 10x restriction buffer
- 'Y' μ l of sterile MQ water
- 'X' μ l of plasmid DNA
- 1 μ l of (each) restriction enzyme

Total 100 μ l

6. Mix by flicking, then tap on bench to get liquid to bottom of tube. Incubate at correct temperature for approx. 15 min. (up to 60 min is OK, but not too much longer than that).

NOTE: 100 μ l seems like a large volume to use, but this helps dilute any impurities in the plasmid prep. Increasing the total volume of the digest and/or reducing the volume of plasmid added often help to improve the quality of a poor digest. If the digested plasmid is for the purpose of ligation, we can use a large volume, since it will be column-purified & concentrated anyway.

DIGEST OF INSERT DNA

1. Calculate how much volume of insert DNA you need to use to get 300 ng – call this A, and calculate how much water to add to the digest; this is $(100-10-2-A)$ – call this B.
2. Label your tube(s). Set up digest by adding ingredients in the following order. Make sure you use excellent aseptic technique, and change tips every time. Its OK to set this up at room temp.

- 10 μ l of 10x restriction buffer
- 'B' μ l of sterile MQ water
- 'A' μ l of insert DNA
- 1 μ l of (each) restriction enzyme

Total 100 μ l

3. Mix by flicking, then tap on bench to get liquid to bottom of tube. Incubate at correct temperature for approx. 15 min (up to 60 min is OK, but not too much longer than that).
4. Dephosphorylate the vector by adding 1 μ l of shrimp alkaline phosphatase (rSAP) enzyme, incubate 30 min at 37°C. This step prevents the vector religating to itself. Do not dephosphorylate both the insert and the vector or nothing will ligate!
5. Purify both vector and insert DNA using silica spin columns, elute in 15 μ l EB.

LIGATION

1. Retrieve one 10x ligase buffer aliquot from the freezer. This should be a small amount of buffer (e.g. 10 μ l) in a small generic tube, not the large tube from the manufacturer with ~1 ml of buffer. Ligase buffer (unlike restriction buffer) cannot be repeatedly frozen and thawed, it starts to 'die' after even one freeze/thaw cycle.
2. Retrieve the T4 ligase enzyme from the -20°C freezer and put IMMEDIATELY on ice. This reagent is VERY heat sensitive, and must be handled with care.
3. Put your labelled tube(s) on ice, then set up the ligase reaction in this tube on ice. Be careful not to get ice or melted ice in the tube - this is not sterile! Add reagents in this order, change tips each time :
 - 2 μ l of 10 x ligase buffer
 - $(17 - X - Y)$ μ l sterile milliQ water
 - X μ l purified insert DNA
 - Y μ l purified plasmid DNA
 - 1 μ l of T4 DNA ligase enzyme

Total 20 μ l

4. Mix by flicking briefly then incubate either 4°C overnight or room temp for 30-60 min. Return the ligase enzyme immediately to -20°C freezer. Throw out any unused thawed ligase buffer.

Note #1. Adding too much DNA can also mean adding excess salt or other junk that can hinder the ligase, ie. more DNA is not always better here.

Note #2. Typically we add 3-fold more insert vs plasmid, on a molar basis. e.g. if you are ligating a 1kb fragment into a 3kb plasmid, 100ng of the plasmid can only accept 33ng of the insert so a three-fold excess of insert is ~100 ng. Having a slight excess of insert DNA makes it more likely that it will bump into the plasmid and the ligase, but too much and it will be more likely just to ligate to other copies of itself, or to give you plasmids with multiple copies of the insert back-to-back.

Protocol for troubleshooting ligations and transformations

NOTES: When you first do a particular ligation, you should include all of the controls described below. Once you are 'up and running', and just repeating an experiment that has worked well before, you can omit most of these, but you should always do at least the positive control (vector only) and the negative control (no DNA added) in any ligation / transformation experiment.

See the related protocol (above) on setting up ligations for the hands-on details of the basic ligation method. This current protocol just focuses on the different ligation controls, and interpreting the results that arise from them. Note that the use of phosphatase in the procedure, and the inclusion of this control is optional if you are using two different restriction enzymes for cloning, but is essential if you are using a single restriction enzyme for cloning.

SUMMARY OF CONTROLS:

1. **Negative (no plasmid) – are the antibiotic plates OK? (expect no colonies)**
2. **Positive (uncut plasmid) – are plasmid stock + comp cells OK? (expect very many colonies)**
3. **Digest (cut plasmid) – are the restriction enzymes working? (expect very few colonies)**
4. **Ligation (cut, religated plasmid) - is the ligation working? (expect many colonies)**
5. **Dephosphorylation (cut, dephos, religated plasmid) – is phosphatase working? (expect few colonies)**

DETAILED EXPLANATIONS:

1. Transformation negative control: 50 µl of competent cells, no DNA added.

This control is primarily to check that you have made the antibiotic agar plates correctly, and it should yield no colonies at all. If you see lots of colonies here, this most commonly means either you forgot to add antibiotic to the plates, or the antibiotic concentration is wrong (too low), or the host bacteria are already resistant to the antibiotic (e.g. TOP10 has chromosomal streptomycin resistance).

Other possibilities could be that the plates were incubated too long (especially with LB-ampicillin), or that there is severe contamination with an antibiotic-resistant bacterium (not E.coli) (this is unlikely!), or that there was a mix up of labelling somewhere e.g. is this really the positive control?

If you see just a few colonies on these plates, this indicates some kind of contamination has occurred during the procedure, e.g. from one of the other samples or the pipette etc. This may not be a 'deal-breaker' so long as there are lots more colonies on your experimental test plates.

2. Positive control: 50 µl of competent cells + 1 µl of plasmid

This control is to check that your stock of the plasmid vector is OK, and that your competent cells are indeed competent; it should yield thousands of colonies on the 'pellet' plate. If you see no growth here, or only a handful of colonies, possibilities are as follows: the cells are not competent, used the wrong antibiotic in the agar (check the sequence of your plasmid to confirm correct resistance), used the wrong concentration of antibiotic (too much), the agar plates are 'bad' for some other reason (e.g. added mercuric chloride instead of sodium chloride!), the plasmid stock has gone bad (run a gel to check), there was a mix up of labelling, or there was a pipetting error (look at the pipette tip to ensure you really have 1 µl of plasmid in there!)

3. Digest controls. 50 µl of competent cells + 3 µl of purified digested plasmid

This control is to check that your restriction enzymes are cutting the plasmid vector effectively. It should yield only a few colonies (approx. < 20 on the 'pellet' plate). If you see hundreds or thousands of colonies here, either the restriction enzyme has gone bad, the digest was set up incorrectly, your plasmid stock is not sufficiently pure, or you have put too much plasmid into the digest. A separate digest control is needed for each enzyme you are using for cloning – e.g. if you are cloning an EcoRI-XbaI fragment into a vector cut with the same enzymes, you need to test EcoRI digestion and XbaI digestion separately.

Set up digests as described above, as if you were going to ligate the plasmid to an insert (ie. 100 ng plasmid in 100 µl digest, then after incubation, purify on column, and elute in 15 µl EB), but don't set up the ligations, just transform the purified, digested plasmid directly into the cells.

The digest controls should be interpreted alongside an agarose gel run with the remainder of the digested, purified plasmid; the latter should give a single sharp band at the expected total size of the vector plasmid. If you see a lot of smearing in this digest this could indicate non-specific nucleases are contaminating the reaction. If you see additional bands in addition to the band at the expected size, this could indicate incomplete digest (this is quite common) – these extra bands are the supercoiled circular and/or open-circular forms of the plasmid – you need to remove these by re-doing the digest with less DNA and/or more-purified DNA and/or a larger volume digest. The uncut plasmid bands tend to look 'fuzzier' than the cut plasmid.

If you have undigested plasmid remaining in the mix, you will get a very high background of vector-only clones, which will make it hard to find your clones of interest. The digest controls will also reveal if your chosen restriction enzymes definitely cut in the expected locations, or if there are other unexpected cut sites in the vector backbone (bad!) – that will give multiple sharp bands, which add up to the expected total plasmid size.

4. Ligation control: 50 µl of competent cells + 3 µl of purified, single-digested, religated plasmid

This control is to check if the ligation step is working. It should yield hundreds of colonies on the 'pellet' plate. If you see only a few colonies or no colonies, this most likely means that either the ligase enzyme is bad or the ligase buffer is bad. Set up digests and ligations as described for the standard ligation procedure, but using a single restriction enzyme only, and no phosphatase step, and no insert DNA. (if you are cloning with two different restriction enzymes, you need to prepare two separate ligation controls, since a double-digest would not be expected to religate in the absence of insert DNA).

This control needs to be interpreted side-by-side with the other controls listed above to ensure that the problem is not poor-quality plasmid DNA or non-competent cells or bad agar plates etc. In addition to bad ligase or bad ligase buffer, this could also be due to bad ligation setup or conditions (e.g. a bad batch of MQ water). Another possibility if the ligation control doesn't work is that there are non-specific nucleases getting into your restriction digest – this would mess up the ends of the DNA and prevent it religating. (check digests on gel; they will look smeary if you have non-specific nucleases).

5. (optional) Dephosphorylation control. 50 µl of competent cells + 3 µl of purified, single-digested, dephosphorylated, religated plasmid

This control is to check that the phosphatase enzyme is working. It should yield very few colonies (approx <20 on the pellet plate). If you see hundreds or thousands of colonies, it means that the phosphatase enzyme or buffer is bad, or you forgot to add phosphatase buffer or enzyme.

Set up the digest, dephosphorylation, and ligation as described for the standard ligation procedure, but using a single restriction enzyme only, and no insert DNA. In this situation, the ligase will attempt to join the cut plasmid backbone to itself, but it should fail to do this since the 5' phosphate groups have been removed. This control must be interpreted alongside the other controls to rule out e.g. lack of restriction digestion if you see many colonies appearing.

Cloning/ligation protocol using heat-killed digests

Notes: This protocol is especially useful for ligations involving very small or large fragments which do not get retained very well during column purifications. It's also worth considering when you don't have much DNA in your sample and want to minimise losing it. Note that some restriction enzymes are not heat-killable (e.g. PstI-HF and BamHI) so check this first ! (NEB website)

The thermostat on the heat-block isn't very accurate and usually the block is 5-10°C below the set point. Check the thermometer before you start. Putting the thermometer in an Eppi tube of water in the block will give you the closest idea of what your sample is actually being heated to.

A downside of this protocol is that it doesn't remove small offcut bits of DNA like column purification does. If for example you are digesting a PCR product that isn't too small, or at two sites in a plasmid with only a few bases between them, it would be better to column purify these digests to reduce the chance of the small fragment re-ligating back in.

Protocol

1. Digest: Set up a restriction digest in a small volume in an Eppi tube. Incubate 37°C for >30 min.

1µL	10x Cutsmart (or other buffer, check first)
1µL	Enzyme 1
1µL	Enzyme 2
x µL	insert DNA
y µL	vector DNA
(7-x-y) µL	MilliQ-H ₂ O

2. Heat kill. While waiting for digest, turn on the heating block and set to whichever is the highest heat kill temperature of the two restriction enzymes. When digest is complete, place the tube with DNA into the heating block for 20 min. Turn off heat block! Chill DNA on ice for 5 min.

3. Ligation. Give the tube a quick spin to get all the liquid to the bottom. Add 1 µl of 10x T4 DNA ligase buffer and 1µL of T4 DNA ligase. Ligation can be done at room temperature for 30 min on your bench or overnight in the cold room.