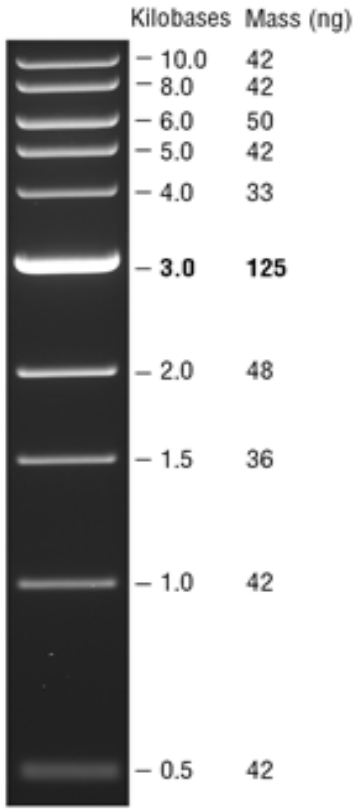
Protocol for quantitation and restriction digestion of plasmid (gel analysis)

1. Quantitate your plasmid via running a small amount (e.g. 1 ul and 5 ul) on an agarose gel. Alongside this, run a few lanes with known amounts of DNA ladder (e.g. 5 ul, 10 ul, and 20 ul of a 1/20 dilution of the NEB 1 kb ladder) to enable comparisons. Make sure you run the gel far enough to separate out all the bands in the ladder (till blue dye is right at the end). Using 0.7% agarose instead of 1% agarose and/or 1xTAE instead of 0.5xTBE can help to resolve large bands in the DNA ladder.



2. Estimate plasmid concentration by finding a band(s) in one of the ladder lanes that corresponds to the band(s) in one of your plasmid dilutions. This is not exact, but is more accurate than using Nanodrop or other UV spectro.– these methods are OK for column-purified PCR products, but overestimate DNA in plasmid preps. The NEB 1 kb ladder is shown (→), note the amounts of DNA in each band are given in ng (assumes you load 0.5 µg = 1 µl of undiluted marker)

3. Choose a restriction enzyme (RE) for the digest that will cut the plasmid 1-3 times. Too many cut sites complicates interpretation. If you use an enzyme that cuts more than once, check that the predicted fragments aren't close in size. It's a good idea to choose an enzyme that you are going to use for subsequent cloning steps – this lets you confirm your enzyme is working OK and it cuts the expected number of times. If you are using two enzymes for the cloning, do two separate digests (you won't usually be able to tell if both of them cut effectively when the RE sites are close together, in the same multiple cloning site)

4. Check the buffer and temperature requirement of the enzyme. Most RE's work best at 37°C but some (eg SmaI) are better at 25°C. Retrieve the 10x buffer from the freezer and thaw this out thoroughly (e.g. in a 37°C waterbath) – its important that the buffer is fully thawed or the salt conc wont be correct. Give the tube a good mix by vortexing or flicking. Then get out the RE itself – transfer immediately to ice – <u>it is critical that the RE stays cold at all times</u>.

5. Set up the digest with ~250 ng plasmid in a 30 µl volume with 2 µl of RE, as follows. First figure out how much plasmid you need – hopefully this is less than ~ 5 µl (lets call this X µl). If X > 5 µl, the digest may not work due to junk in the plasmid prep (e.g. salts) interfering – in this case, consider further purification and concentration of the plasmid e.g. by ethanol precipitation. Calculate the amount of water (sterile Milli-Q; MQ) to add to the digest; this is (30 – 3 – 2 – X); lets call this Y. Set up the digest in a sterile Eppi tube in this order:

- 3 µl of 10x buffer
- Y ul of MQ
- X ul of plasmid
- 2 ul of RE

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total 30 µl

With any molecular biology reaction, you usually add the enzyme **last**. Don't add enzyme to plain MQ or to 10x buffer. Be careful not to contaminate the enzyme with DNA, and vice-versa ! Change tips for every addition !

6. Incubate at the preferred temperature for ~2 hours. There is a wide acceptable time range here (30 min – 24 h). 2 hr is a compromise between allowing maximum digestion by the specific RE, and minimising non-specific digestion by traces of other nucleases. Run on agarose gel to check.