PCR

1. Combine in a 0.2ml microcentrifuge tube:
    - 5µl of NEB 10X standard Taq buffer (final concentration = 1X)
    - 0.25µl of NEB Taq (final concentration = 1.25U/50µl)
    - 1µl of 10µM forward primer (final concentration = 0.2µM)
        i. Reduce [primer] to reduce non-specific binding to undesired sites (0.1-0.15 uM; 0.5 uL-0.75 uL of 10 uM primers)
    - 1µl of 10µM reverse primer (final concentration = 0.2µM)
    - 1-2uL of template plasmid DNA (final concentration < 1000ng/µl)
    - 1µl of 10mM Kapa dNTPs (final concentration = 200µM)
    - Ultra-pure PCR grade, RNA-free H_2O to 50µl
2. Vortex 2 to 3 seconds to mix, then centrifuge briefly to settle
3. Place in thermocycler and select or set up the appropriate program as follows:
    - Initial denaturation -> 95°C for 30 seconds
    - Repeat 25 to 30x:
        i. Denaturation -> 95°C for 15 to 30 seconds
        ii. Annealing -> T_m - 5°C (45 to 68°C) for 15 to 60 seconds
        iii. Extension -> 68°C for 1 minute per kilobase
    - Final extension -> 68°C for 5 minutes
    - Hold at 4°C

**DO NOT hold at 4ºC overnight (this can reduce the lifespan of the thermocycler), DNA can be held at ≥10ºC (even room temperature) without degrading

Use of normal PCR in the lab:

1. MAIN use in iGEM= amplifying gBlocks
    a. PCR product is run on gel and gel purified → you can lose a lot of DNA through this method
        i. PCR Purification may give you higher yields:
            1. Take a piece of parafilm
            2. Spot 2µL of 6X gel loading dye on the parafilm (spot 2µL for each of your PCR samples)
            3. Mix 10µL of PCR product with the 2µL of loading dye on the parafilm
            4. Load the mixed sample on the gel
            5. If you get a single clear band at the expected size proceed with a PCR purification with your remaining PCR product. If you get multiple unclear bands proceed with a gel purification (and run more of your PCR product on a gel to gel purify)

2. Overhang PCR= Design primers with a 5' overhang (3' end anneals to your target) if you want to add a sequence to the 5' end (this can be used to add a restriction site or gibson assembly overhang, or any other sequence you need)
    a. You will have to play around the PCR settings (especially annealing temperature) to make sure the PCR works. Running a temperature gradient for annealing temperature (if your thermocycler is capable) would help

3. Site-directed mutagenesis= Design primers with single nucleotide changes to make single-nucleotide changes in your target DNA

Troubleshooting tips:

NOTE: PCR can routinely fail and sometimes requires a lot of troubleshooting (the same mastermix and thermocycler protocol DOES NOT work for everything → we tend to use the same saved PCR thermocycler protocol and make mastermixes in bulk, but be prepared to revise/optimize these if necessary)

1. PCR mastermixes can "go bad" if stored in the freezer for too long → making it fresh may give you more chance of success
2. Addition of 0-5% DMSO (depending on the taq polymerase you're using) in the mastermix can help with particularly GC rich DNA templates (but this may change the optimal annealing temperature)
3. Optimize the concentration of primers, dNTPs, and Taq polymerase based on the taq polymerase being used
    a. Check the protocol from the manufacturer of the Taq polymerase you're using
    b. Decreasing primer concentration can sometimes help (minimum ~0.2µM)
        i. Decreases off-target binding
        ii. Decreases primer-primer interactions
4. Increasing the amount of template DNA being used
5. Extension time is not sufficient (remember 1 minute per kb→ look at manufacturer's protocol)
6. Extension temperature (and denaturation temperature) are not optimal → this will vary based on the type of Taq polymerase you're using
    a. I believe we're using KAPA Taq right now which has different PCR conditions than those listed in the protocol above: KAPA Taq protocol link
7. Use PCR water (should be Ultra-Pure and RNase free)