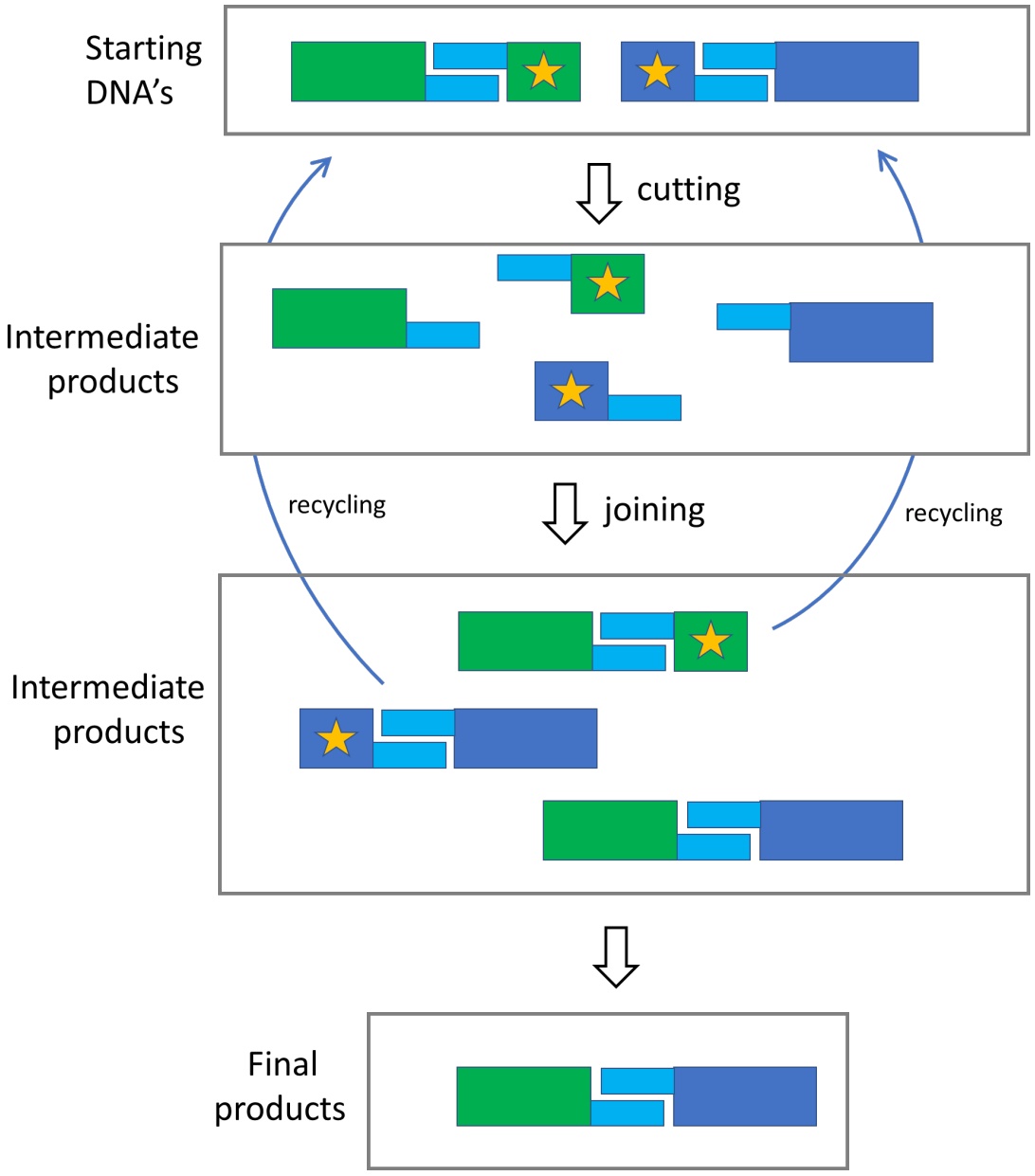
Protocol for Golden Gate assembly

Golden Gate assembly is useful for seamless assembly of multiple DNA fragments. It takes advantage of the separate recognition and cut sites of Type IIS restriction enzymes (BsaI, Esp3I). This separation of recognition site vs. cut site and has two consequences. Firstly, it means the cut/join site can be customised to any four bases, allowing assembly of many fragments. Secondly, it means that restriction enzyme and ligase can be added *together* in the same tube, and thus any correctly assembled products stay joined, but if the original sequences rejoin to themselves, they get cut again; this makes the reaction much more efficient in producing recombinants relative to a normal restriction / ligation reaction (see diagram below, in which the restriction enzyme recognition site is show as a star).



Golden Gate assembly can be used to ligate DNA chunks with or without a plasmid backbone. Fragments can be purified PCR products, purified restriction digests, gBlocks, or any combination of these. These protocols work for any Type IIS restriction enzyme, such as BsaI-HF-v2 or Esp3I. The enzyme BsaI-HF-v2 is optimised for use in ligase buffer, so is the best initial choice.

CREATING AN EQUIMOLAR MIXTURE OF FRAGMENTS

To begin, you need to create an equimolar mixture of all the fragments you will be assembling in the reaction. Create a table in Excel to allow for easy calculations. The order of your inserts in the table does not matter. The first row in the table would typically be the plasmid backbone, if applicable. If you are not using a backbone, this will act as the reference fragment. The aim is to calculate the relative number of copies of each insert relative to the backbone / reference.

Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*
DNA	**No. of bases (kb)**	**[DNA] (ng/µL)**	**ng/kb**	**ng required**	**volume required**	**normalised vol. required**
Backbone or fragment 1					1 µl	
Fragment 2			n/a			
Fragment 3			n/a			
Fragment 4			n/a			
Fragment 5			n/a			
					total vol:	**X** µl

Column 2: You should have this information from SnapGene

Column 3: Use the nanodrop to estimate the concentration of DNA for each insert. If you are cloning with gBlocks, the typical concentration will be 10 ng/µL

Column 4: Divide the DNA conc. by the number of kb for each fragment. This 'ng/kb' value is *kind of* like the number of moles of the DNA present (in reality, it's not accurate due to the different MW of the different bases, but its close enough for this purpose).

Column 5: For each row except the first one, multiply the number of kb of that fragment by the 'ng/kb' value for the backbone / reference.

Column 6: Calculate the volume of each insert needed by dividing the number of ng (column 5) by the concentration (column 3). Note that for the backbone/reference, this value is 1µL.

Column 7: If any the calculated volumes are too small to measure (<1 µl), scale all of them up by the same multiplication factor so that the smallest volume is 1 µl.

For most GoldenGate reactions, the simple protocol below should work well. If this fails, try the full protocol (the pre-cut and clean-up steps in that protocol reduce background parent plasmid). Pre-digesting any PCR products with DpnI can also help to minimise plasmid background in those samples (but don't do this digest on your vector backbone if this is not a PCR product!)

SETTING UP THE GOLDEN GATE REACTION – QUICK PROTOCOL

Assemble the components below in a sterile tube, adding the enzymes last. Keep the enzymes cold at all times, and return to the freezer promptly. Mix well by pipetting up and down a few times, and then incubate at 37°C for 1 hour. You can stop the reaction by heating to 65°C for 10 minutes if you don't plan to transform it into cells immediately. Transform as usual for a ligation mixture.

10 X T4 ligase buffer	2uL
Parts mix	**X** uL (from previous table)
MilliQ water	(16 – X) uL
BsaI-HF v2	1uL
T4 ligase	1uL
Total	20uL

SETTING UP GOLDEN GATE REACTION – FULL PROTOCOL

This protocol is designed to take account of the different optimal temperatures of restriction enzymes (37°C) and ligase (16°C), via thermocycling between these temperatures. A typical reaction takes about 2 hours. The reagents are added in three stages. Assemble the 'Pre-cut' reaction in a PCR tube. Place into thermocycler and do 'phase 1' cycling (see below). Add the 'Ligation' reagents, then do 'phase 2' cycling. Add clean up reagents, then do 'phase 3' cycling.

	Pre-cut (µL)	**Ligation (µL)**	**Clean up (µL)**
CutSmart 10X	1		
CutSmart 5X		1	1
ATP (10 mM)		1.5	
BsaI or Esp3I	1		1
T4 ligase		1	
MQ H_2O		1.5	3
Parts mixture	8		
Total:	10	5	5

THERMOCYCLING CONDITIONS*

Phase 1 cycling**:** 37°C for 10 mins

Phase 2 cycling: 15 cycles of [37°C for 2 mins, 16°C for 2 mins], then 75°C for 15 mins

Phase 3 cycling**:** 37°C for 15 mins

* these conditions assume you are 'on duty' and being careful to extract the samples when each phase finishes (this is best practice). If you must add in hold times for the second two phases, use a 4°C hold for phase 2, and a 37°C hold for phase 3, but try to keep these times to a minimum.