

Supplementary File S1. Protocol and guidelines for DNA-DNA hybridization (DDH).

(A modification of the method of Goris et al. [33]).

- A. Planning:** In our experiments, the DNA of four strains is normally coated onto a single microplate. One strain is coated per strip of 16 wells. One strip of 16 wells is reserved for coating the blank (salmon sperm DNA). The strain numbers are marked on the strips. The amount of DNA to be coated is determined by the amount of probe to be used. If four strains are coated, there will usually be four probes. In that case, the amount of DNA needed per strain for coating will be: 4 probes x 4 wells/probe x 100 μ l DNA/well = 1600 μ l DNA (OD0.2) needed.

Probe DNA: Initially 10 μ l DNA (OD10), equivalent to 5 μ g of DNA, is needed per strain for the probe. Through the dilutions to follow [33] there will be sufficient probe DNA for hybridizations with the probe's own coated DNA, the blank, and the coated DNA of three other strains (4 wells per probe). In the case of four strains being coated, as well as the blank, the amount of wells to fill with one specific probe will be: 5 coated DNAs x 4 wells/probe = 20 wells per probe. It is important to include a positive (homologous) reaction, which is the probe hybridized against its own DNA. It is equally important to hybridize each probe against the blank for background correction. Reciprocal reactions (A x B and B x A) are needed to interpret the results.

In order to achieve comparable results, it is vital to perform all DDH experiments with the same method under identical experimental conditions. The DDH values reported should be the means of at least two independent experiments. The four wells of each specific hybridization reaction in an experiment, will serve as four replicates and the mean value is used for calculations.

- B. Beforehand:** The total genomic DNA extracted from 700-1000 mg of bacterial cells [55; 16] can be very concentrated. The extracted DNA should therefore be left to stabilize at 4°C in 0.1 x SSC for a few days following the extraction. The DNA is stabilized once there is no more variation in the optical density (OD) readings measured on a daily basis. Only at this stage is it feasible to start with the required dilutions.

The quality of the DNA is equally important. The DNA should be very pure and of a high-molecular mass. The size of the DNA and the possible presence of RNA can be checked on a 1% agarose gel. It is of utmost importance to confirm that the DNA is intact and not degraded, which can lead to erroneous results. Purity is confirmed spectrophotometrically using the ratios 260/280 and 260/230. The ideal values are

between 1.8-2.0 and 1.7-2.5 respectively [16]. Any RNA or chemical contaminants in the DNA solution can affect the hybridizations.

Once suitably DNA is available, the first step is to prepare approximately 500 µg of OD10 working solutions for each sample (in 0.1 x SSC) [33]. The original, concentrated stocks can be kept at -20°C for long periods of time if it is not thawed and frozen repeatedly. The OD10 solutions can be kept at 4°C until the experiment is completed, after which it can also be stored at -20°C. Measure the OD of the working solutions very accurately, as the OD10 solutions will also be used for the probes.

C. Coating (immobilization) of DNA in the wells: Polystyrene microplates (MaxiSorp, F16 black, FluoroNunc; Nunc 475515; Thermo Scientific) are used for hybridization. The layout should be planned beforehand and the strips marked.

1. Dilute the OD10 working solutions, as well as the blank, with 0.1 x SSC to obtain 200 µl of an OD2 solution per sample (if one strip is to be coated per sample). Measure the optical density again to confirm the concentration. 5 µl of the OD2 solution can be subjected to 1% agarose gel electrophoresis to confirm the quality of the DNA if it has not been checked recently.
2. The DNA is denatured for 10 min at 100°C, where after it is cooled rapidly in ice water for about 10 min.
3. The DNA is then diluted with 1 x PBS-100 mM MgCl₂ to obtain 2 ml of an OD0.2 solution. Do not vortex, but invert the solution to mix. Measure the OD again to ensure that all DNA solutions have exactly the same concentration.
4. Dispense 100 µl of DNA solution (= 1 µg DNA) very precisely to each well according to the planned plate layout.
5. The microplate is then placed in a hybridization oven to dry overnight at 30°C. Plates are not sealed, but left open for the DNA solutions in the wells to dry completely. This ensures maximum and exact binding of the DNA in the solution, minimizing variation between wells and improving repeatability between DDH experiments. Do not stack plates if more than one plate is used.
6. Proceed with hybridizations immediately the next morning.

D. DNA-DNA hybridization: Before proceeding, the ODs of the working stocks should be checked once more to ensure that the OD is 10 and that all concentrations are equal. The hybridization temperature is calculated as described by Goris et al. [33]. A 400 W lamp fitted with thermal cut-out (Thorlux RS 579-253) is used for photobiotin labelling.

Labelling of probe DNA with photobiotin:

1. Adjust the hybridization oven to the required hybridization temperature.
2. Adjust a heating block to 100°C.

3. Thaw the photobiotin working solution (0.5 mg/ml)
4. Switch on the 400 W lamp about 15 min before use, in order for it to warm up and reach full intensity.
5. Prepare a container with ice and add a metallic cooling block. This should fit easily under the lamp.
6. For each probe take 10 μ l of the OD10 DNA working solution (= 5 μ g DNA) and add 10 μ l photobiotin solution in a 1.5 ml Eppendorf tube.
7. Mix the solution by flicking the tube with one's finger, centrifuge it briefly and put it in the metallic cooling block with the lid open.
8. Put the container with the cooling block approximately 12-15 cm under the lamp in such a way that the Eppendorf tubes are centrally under the lamp. Illuminate for 30 min. Fill up the ice if it starts to melt, in order to prevent evaporation and keep the samples cool.
9. While the DNA is being labelled under the lamp, add 200 μ l pre-hybridization solution to the coated and dried wells of the microplate. Seal the plate with the appropriate vinyl tape (Nunc Amplification tape – Cat. No. 232702 Thermo Scientific) and incubate it in the hybridization oven at the hybridization temperature for at least 30 min.
10. After the illumination, the probe DNA is diluted with 185 μ l 0.1 M Tris/HCl (pH 9.0) and mixed by ticking.
11. Photobiotin that has not been incorporated is removed by butanol extractions: 200 μ l 1-butanol saturated with 0.1 M Tris/HCl is added to the probe mixture, mixed by inverting, centrifuged briefly and the upper butanol phase removed. Repeat the extraction one more time. No more red colour from the photobiotin should be seen in the lower phase.
12. The labelled probe DNA is fragmented by sonication using a Microson™ Cell Disruptor, set at 45 W continuous output for 30 seconds per sample. Keep the samples on ice during the process.
13. Probe DNA is denatured in the heating block at 100°C for 10 min. Immediately cool on ice for at least 10 min. Keep on ice till ready to continue.

Hybridization:

1. In order to fill 20 wells per probe, 2 ml of hybridization mixture (hybridization solution with probe DNA) is needed. 100 μ l of the prepared probe is added to 1900 μ l of hybridization solution, the latter preheated at the hybridization temperature.
2. Remove the pre-hybridization solution from the plate by flicking it over. Blot it on towel paper to dry excess liquid.
3. Add 100 μ l hybridization mixture (containing 1 μ l probe DNA/ml) to the wells according to the pre-determined layout. A specific probe should be applied in the same quadrant of the different strips.

4. Each probe has to be added to 4 wells of the blank, four wells of its own coated DNA, and four wells each of the other three coated samples. Pipette very precisely and avoid bubbles in the wells.
5. Seal the plate with vinyl tape and incubate for 3 h at the specific hybridization temperature.

E. Enzymic development and quantification:

1. Prepare the streptavidin- β -galactosidase and 4-methylumbelliferyl β -D-galactopyranoside (4-MUF) solutions before the 3 h hybridization period is over.
2. Take the plate out of the oven and adjust the temperature of the oven to 37°C. Also put an empty, used plate in the oven.
3. Using a microplate washer (Wellwash AC microplate washer - Thermo Electron Corporation), wash the plate three times with 300 μ l 1 x SSC per well and leave the wells dry.
4. Add 100 μ l of streptavidin- β -galactosidase working solution to the wells.
5. Incubate the microplate for 15 min at 37°C, covered with the empty preheated plate.
6. Wash the plate three times with 300 μ l 1 x SSC per well with the microplate washer.
7. Set up the microplate reader (Fluoroscan Ascent microplate fluorometer – Thermo Electron Corporation: wavelength in (excitation), 360 nm; wavelength out (emission), 465 nm).
8. Add 100 μ l of 4-MUF (acting as the substrate for streptavidin- β -galactosidase) to each well. The reaction product, 4-methylumbelliferone (excitation maximum 360 nm; emission maximum 465 nm) is quantified using the microplate fluorometer.
9. Immediately start measuring (time = 0 min). Continue to read at 15, 30 and 45 min. Incubate the microplate at 37°C between readings, covered with an empty microplate.
10. The DDH values are calculated using the fluorometer measurements at 30 or 45 min. Readings are checked visually and clearly aberrant values omitted.

F. Calculation of % DNA similarity

- Data is transferred to an Excel sheet with columns corresponding to each strip on the plate.
- The measurements obtained for each well is entered according to its position on the plate.
- The average of the fluorescence values of each quarter (values for one probe) is calculated for all the strips.
- The blank value obtained for each probe (probe x immobilized salmon sperm DNA) is subtracted from the values of its corresponding hybridization reactions.

- Homologous reactions are regarded as 100% reassociation. The rest of the hybridization values for each probe are calculated as a ratio to the value obtained in the homologous reaction and expressed as a % DNA similarity.
- The values for the reciprocal reactions (different hybridizations using the same DNAs, A and B, but once with A as the immobilized DNA and once with B as the immobilized DNA [33] are used to calculate the mean % DNA similarity for a specific pair of DNA. The difference between the reciprocal reactions is divided by two and reported with the mean value as a \pm value in parenthesis).

Materials [33]

1. 10 x SSC (standard saline citrate)
 - 87.7 g NaCl
 - 31.5 g Citric acid
 - 18 g NaOH
 - pH 7.0 met NaOH
 - Add milliQ water up to 1000 ml
 - Store at 4°C
2. 10 x PBS (phosphate-buffered saline)
 - 40 g NaCl
 - 1 g KCl
 - 5.75 g Na₂HPO₄·2H₂O
 - 1 g KH₂PO₄
 - pH 7.2 met NaOH
 - Add milliQ water up to 1000 ml
 - Store at 4°C
3. 1 x PBS-100mM MgCl₂
 - 50 ml 10 x PBS
 - 10.15 g MgCl₂·6H₂O
 - Add milliQ water up to 1000 ml
 - Filter sterilize
 - Store at 4°C
4. Pre-hybridization solution (500 ml)
 - 100 ml 10 x SSC
 - 50 ml 50 x Denhardt solution
 - 5 ml low molecular weight denatured salmon sperm DNA (10 mg/ml)

- 95 ml milliQ water
 - 250 ml formamide (in order to work under stringent conditions at a lower temperature)
 - Store at -20°C
5. Hybridization solution (100ml)
- 100 ml pre-hybridization solution
 - 2.5 g dextran sulphate (Sigma) (to accelerate the hybridization)
 - Add stirring bar to dissolve (takes approx. 30 minutes)
 - Store at -20°C
6. 0.1 M Tris HCl
- 2.422 g Tris (MW 121.1)
 - pH 9.0 met HCl
 - Add milliQ water up to 200 ml
 - Filter sterilize
 - Store at 4°C
7. 1-butanol saturated with 0.1 M Tris HCl
- Add 0.1 M Tris HCL (pH 9.0) to 1- butanol for molecular biology (Sigma).
 - Shake well and leave until two phases are formed.
 - The top phase will be butanol.
8. Photobiotin stock (Sigma A1935) – acetate salt
- Dissolve the photobiotin powder (1 mg) in 1 ml of water (stock solution).
 - Dilute it to 0.5 mg/ml in water for the working solution.
 - Wrap both solutions in aluminium foil to protect it against light.
 - Store both solutions at -20°C.
9. Streptavidin- β -D-galactosidase stock
- Dissolve 500 units of streptavidin- β -D-galactosidase conjugate (Roche Cat. No. 11 112 481 001) in 500 μ l milliQ water.
 - Store in aliquots at -20°C until the expiration date.
 - Avoid repeated freezing and thawing.
10. Streptavidin- β -D-galactosidase working solution (per plate)
- 0.05 g BSA (Albumin from bovine serum – Sigma)
 - 1 ml 10 x PBS
 - 9 ml milliQ water
 - 10 μ l Streptavidin- β -D-galactosidase stock (from the prepared aliquots)

- Prepare fresh shortly before dispensing it in the wells.
11. MUF stock (10 mg/ml in dimethylformamide)
- Dissolve 10 mg MUF (4-methylumbelliferyl β -D- galactopyranoside) Sigma (stored at -20°C) in N,N-dimethylformamide (Sigma) so that the concentration is exactly 10mg/ml.
 - Wrap in aluminium foil as it is sensitive to light.
 - Store at 4°C
 - Prepare fresh stock solutions very regularly or freshly prepare a smaller volume for each experiment.
12. MUF working solution (per plate)
- 2.03 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
 - 1 ml 10 x PBS
 - 9 ml milliQ water
 - 100 μl 4-MUF stock
 - Prepare fresh shortly before dispensing it in the wells and keep on ice.
 - Wrap in aluminium foil as it is sensitive to light.