GISH Protocols

Isolation of Plant Genomic DNA for GISH

1. Grind 5 g plant leaves in liquid nitrogen. Never let the plant material thaw. Pour powder into a 250 ml flask.
2. Suspend powdered material in 15 ml of 2X CTAB buffer and incubate in a water bath at 65°C for 1 hr at slow shaking.
3. Let the flasks cool down to room temperature. Add 15 ml chloroform/isoamyl alcohol (24:1) and swirl the flask until it makes an emulsion (place the flask on a rotary shaker 15 min -1 hr at room temperature).
4. Pour sample into a centrifuge tube and spin at 10000 rpm for 15 min at room temperature.
5. Transfer the supernatant to a clean tube and add 2/3 volumes of isopropyl alcohol. Invert the tube several times. Hook out the precipitated DNA and transfer it to a clean 10 ml tube.
6. Wash the DNA pellet twice with 70% ethanol. Pour off the ethanol and let the pellet air dry for 1 hr.
7. Add 500 µl of TE and transfer the DNA into a 1.5 ml tube. Add 5 µl RNase A (10 mg/ml) and leave at 4°C overnight or 37°C for 1 hr.
8. Add 500 µl phenol, mix well, spin for 5 min. Transfer the supernatant to another tube.
9. Add 250 µl each of phenol and chloroform, mix well, spin for 5 min. Transfer the supernatant to another tube.
10. Add 500 µl chloroform, mix well, spin 5 min. Transfer the supernatant to a 10 ml tube.
11. Add TE to 3 ml then add 1/10 volume of 3M NaAc and 2 volumes of 100% ethanol and invert the tube several times.
12. Wash the precipitated DNA twice with 70% ethanol. Transfer the DNA to a 1.5 ml tube. Dry the DNA pellet and dissolve into 500 µl TE.
13. Determine the DNA concentration by running a mini-gel.

Notes:

a. 2X CTAB buffer: 1.4 M Nacl; 100 mM Tris ph 8.0; 2% CTAB (Hexadecyltrimethylamonium bromide); 20 mM EDTA; 0.5% Na Bisulfite; 1% 2-mercaptoethanol (2-me).

b. When making the CTAB buffer it is fastest to add the NaCl after the other ingredients (except the 2-me) are in solution. Do not add the 2-me until just before use. The solution without the 2-me can be stored at room temperature.

c. For genomic in situ hybridization (GISH) purpose, the cleaner the genomic DNA, the better the biotin-labelled probe. Therefore, the isolated DNA may be further purified by CsCl purification procedures. However, the quality of DNA isolated with above protocol is good enough for GISH in our hands.

Preparation of Genomic DNA for Blocking

1. Add 10 M NaOH in the DNA sample (0.1-1 ug/ul) to a final concentration of NaOH of 0.4 M.
2. Place the DNA sample (in microtube) in boiling water for 40 to 45 min.
3. Cool the DNA sample on ice. Add equal volume of 3M NaAc (pH 4.6) and two volumes of 100% ETOH, mix the sample well.
4. Centrifuge for 10 min, rinse the pellet with 70% ETOH, drain the pellet well.
5. Add certain volume of TE, add 1/10 volume of 3M NaAc (pH 4.6) and two volumes of 100% ETOH, mix the sample well, then repeat step 4.
6. Add certain volume of TE, add 1/10 volume of 3M NaAc (pH 7) and two volumes of 100% ETOH, mix the sample well.
7. Centrifuge for 10 min, drain the tube and rinse pellet with 70% ETOH, dry the pellet, dissolve in the final volume of TE.

Notes:

a. The final volume (concentration) of the DNA sample is very important for genomic in situ hybridization or genomic blocking (differentiation) analysis. You may test several times (concentrating or diluting the sample) to get the perfect concentration. For detecting the alien chromosome in a wheat-alien addition line, the wheat blocking DNA should be 50-300 times more than the probe DNA in hybridization mixture.

b. You can use other techniques to prepare the blocking DNA, such as autoclaving, shearing the DNA using a syringe and a small needle, or sonicating. The advantages of this techniques include: i) it is easy to control the size of the DNA (size range from 100 bp to 1 kb with 40-45 min boiling). ii) the DNA sample will be clean because of the repeated precipitation.

Biotin/digoxigenin-labelling of Plasmid or Genomic DNA

1. Prepare following reaction solution:

10X nick-translation buffer	5 µl
dNTP solution	2.5 µl
dUTP solution (biotin or digoxigenin)	2.5 µl
Plasmid or Genomic DNA	1 µg
DNA Polymerase I	12 units
DNase I	
H2O	
Reaction volume (total)	50 µl

2. Use H2O to adjust the total volume to 50 µl. Carefully mix after each step of adding solutions and spin down at the end.
3. Incubate reaction tube in water bath at 14-15˚C for 2 hrs.
4. Stop the reaction by adding 5 µl stop buffer (200 mM EDTA).

Notes:

a. 10X nick-translation buffer: 0.5 M Tris HCl pH 7.5, 50 mM MgCl2. dNTP solution: containing 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP. dUTP solution: 0.166 mM dUTP (biotin or digoxigenin-congugated) and 0.333 mM dTTP .

b. The most critical part for the labelling (if the DNA is clean) is the size of the final nick translation product. The best in situ hybridization results will be obtained when the size of the biotin-labeled probe is around 200-600 bp.
c. For determining the size of the probe, denature 15 µl of the nick translation product in boiling water for 4 min and cool down on ice immediately. Run the sample on a mini-gel with a DNA size marker.
d. The size of the nick translation product can be adjusted by adding different amount of DNase I in the reaction solution.
e. Random priming technique can also be used for labelling. Since the size of probe is relatively easier to control by nick translation, we prefer to use the nick translation technique.
f. We suggest you to buy a nick-translation kit if you don't have enough experience. Several companies sell such kits which include the biotin-dUTP or digoxigenin-dUTP.

II. Probe Purification

1. Start 20 min before the end of the nick translation incubation. Plug the bottom of a 1 ml tuberculin syringe with siliconized glass wool.
2. Fill the syringe with Sephadex G-50 up to the top using a sterilized pasteur pipette.
3. Place the Sephadex-filled syringe into a 15 ml Corex tube.
4. Centrifuge at 1500 rpm for 4 min to pack down the sephadex.
5. Repeat step 2 to 4 so that the packed sephadex column has a total volume of about 0.9 ml.
6. Wash the column twice with 55 µl TE by spin at 1500 rpm for 4 min each.
7. Place a sterilized 1.5 ml tube on the bottom of the 15 ml Corex tube, insert the packed and washed syringe into the corex tube such that the tip of the syringe is fitted into the 1.5 ml tube. Load the nick translation product (55 µl, with stop buffer) and spin at 1500 rpm for 4 min.
8. The probe DNA will be collected in the 1.5 ml tube.

Notes:

a. If you did not make any mistakes, the final volume should be close to 55 µl. Usually 0.5 µl of such biotin-labelled probe (1 µg/55 µl = 18 ng/µl) is enough for each slide for in situ hybridization. Thus you can use the probe for at least 100 slides.
b. You can check the size of the probe (by running a mini-gel) either before or after the spin-column purification.
c. The probe can be stored in a freezer for several years. You may store it at 4°C if you use it regularly.
d. The spin-column method can be replaced by other techniques for probe purification. You can use an easier ethanol precipitation method.

Hybridization of the Probe to Chromosomes

1. Remove the cover slips of the slides (stored in a -80°C freezer) with a razor blade and dry slides in an ethanol series (70%, 95%, 100% ethanol 5 min each at room temperature). The slides should be dried several hrs before in situ hybridization.
2. Prepare the hybridization mixture as follows:

deionized formamide	10 µl
20X SSC	2 µl
Sheared salmon sperm DNA (10 mg/ml)	2 µl
Probe DNA	1-2 µl
H2O	? µl
50% dextran sulfate	4 µl
Total	20 µl

Notes: Use H2O to adjust the total volume to 20 µl. 10 µl will be applied to each slide with a 18 ´ 18 mm cover slip. Make sure the solution is well mixed because the 50% dextran sulfate is very sticky.

3. Denature this mixture by placing at a 80°C heating block for 5 minutes and immediately chill the mixture on ice. Spin down the solution.
4. Add 150 µl of 70% formamide in 2X SSC solution on the dried slides and cover the slides with 22 ´ 40 mm cover slips. Place slides on a plate (Metal or glass) in a 80°C hot oven for 1.5 min. (10 ml stock solution of 70% formamide in 2X SSC: 7 ml formamide, 1 ml 20X SSC, 2 ml H2O)
5. Remove the cover slips by swing the slides and dip the slides immediately into an cold ethanol series (70%, 95%, and 100%, 5 min each at -20°C) and air dry the slides.
6. Apply 10 µl hybridization mixture to each slide and cover with a 18 ´ 18 mm cover slip. Seal the cover slip with rubber cement and place the slides in a wet chamber.

Notes: You can use any kind of boxes with 2 layers of wet 3 mm Whatman paper on the bottom, use plastic bars or something else to hold the slides.

7. Incubate the wet chamber at 37°C for minimum 6 hrs or overnight.

Notes:

a. You may check the dried slides under a phase contrast microscope to make sure the metaphase cells are still there and the chromosomes are still in good shape (flat). This is very important. You will never get satisfactory results if the chromosomes become ugly after drying.

b. The quality of formamide is important. The formamide from CMS is very good in our hands.

Detection of ISH Signals with Fluorochromes

1. Peel the rubber cement using a forceps. Dip the slides in a staining jar containing 2X SSC. Gently shake the staining jar until the coverslips fall from the slides.
2. Wash the slides with the following steps:

2X SSC, room temperature	5 min
2X SSC, 42°C	10 min
2X SSC, room temperature	5 min
1X PBS, room temperature	5 min

Notes: You may increase the temperature and time of the 2X SSC wash if you want to reduce background. The above washing series works fine for most of the probes in our hands.

3. Drain (never dry) the slides on paper towels and add 100 µl of FITC (or rhodamine) conjugated anti-biotin (or anti-digoxigenin) antibody (1:50 to 1:200 dilution depending on the product from different companies) cover with 22 ´ 40 mm coverslips, incubate at 37°C for 30 min.
4. Remove the coverslips by tilting the slides or dipping the slides in a beaker with 1X PBS. Wash the slides three times in 1X PBS (5 min each) at room temperature. Notes: You can change the time and temperature for the washing to adjust the stringency.
5. Drain the slides on paper towel. Add a thin layer of antifade solution (from Vector) containing 1 µg/ml propidium iodide or DAPI (4',6-diamidino-2-phenylindole), cover with a coverslip.
6. Check your in situ hybridization results using a fluorescence microscope. The FITC-detected probes will be in a yellow-green color. The rhodamine-detected probes will be a red color.