

Nucleic Acid Hybridization – General Aspects

This chapter discusses the effects of various components of the hybridization solution on the rate of renaturation and thermal stability of DNA hybrids free in solution. The features will be more or less identical to those of immobilized nucleic acids, such as in filter and *in situ* hybridizations. The largest deviation probably occurs in the kinetics. The reader is referred to the following literature for more background information: Casey and Davidson (1976), Cox et al. (1984), Flavell et al. (1974), Hames and Higgins (1985), Maniatis et al. (1982), Raap et al. (1986), Schildkraut and Lifson (1965), Spiegelman et al. (1973), Wetmur and Davidson (1968), Wetmur (1975).

The main parameters that influence hybridization

Hybridization depends on the ability of denatured DNA to reanneal with complementary strands in an environment just below their melting point (T_m).

The T_m is the temperature at which half the DNA is present in a single-stranded (denatured) form. The T_m value is different for genomic DNA isolated from various organisms, e.g., for *Pneumococcus* DNA it is 85°C, for *Serratia* DNA it is 94°C. The T_m can be calculated by measuring the absorption of ultraviolet light at 260 nm. The stability of the DNA is directly dependent on the GC content. The higher the molar ratio of GC pairs in a DNA, the higher the melting point.

T_m and renaturation of DNA are primarily influenced by four parameters:

- ▶ Temperature
- ▶ pH
- ▶ Concentration of monovalent cations
- ▶ Presence of organic solvents

Temperature

The maximum rate of renaturation (hybridization) of DNA is at 25°C. However, the bell-shaped curve relating renaturation rate and temperature is broad, with a rather flat maximum from about 16°C to 32°C below T_m .

pH

From pH 5–9, the rate of renaturation is fairly independent of pH. Buffers containing 20–50 mM phosphate, pH 6.5–7.5 are frequently used.



Higher pH can be used to produce more stringent hybridization conditions.

Monovalent cations

Monovalent cations (*e.g.*, sodium ions) interact electrostatically with nucleic acids (mainly at the phosphate groups) so that the electrostatic repulsion between the two strands of the duplex decreases with increasing salt concentration, *i.e.* higher salt concentrations increase the stability of the hybrid. Low sodium concentrations affect the T_m , as well as the renaturation rate, drastically.

Sodium ion (Na^+) concentrations above 0.4 M only slightly affect the rate of renaturation and the melting temperature.

The following equation has been given for the dependence of T_m on the GC content and the salt concentration (for salt concentrations from 0.01 to 0.20 M):

$$T_m = 16.6 \log M + 0.41 (\text{GC}) + 81.5$$

where M is the salt concentration (molar) and GC, the molar percentage of guanine plus cytosine. Above 0.4 M Na^+ , the following formula holds:

$$T_m = 81.5 + 0.41 (\text{GC})$$

Free divalent cations strongly stabilize duplex DNA. Remove them from the hybridization mixture or complex them (*e.g.*, with agents like citrate or EDTA).

Formamide

DNA melts (denatures) at 90°–100°C in 0.1–0.2 M Na^+ . For *in situ* hybridization this implies that microscopic preparations must be hybridized at 65°–75°C for prolonged periods. This may lead to deterioration of morphology. Fortunately, organic solvents reduce the thermal stability of double-stranded polynucleotides, so that hybridization can be performed at lower temperatures in the presence of formamide.

Formamide has for years been the organic solvent of choice. It reduces the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72°C for each percent formamide. Thus, hybridization can be performed at 30°–45°C with 50% formamide present in the hybridization mixture. The rate of renaturation decreases in the presence of formamide. The melting temperature of hybrids in the presence of formamide can be calculated according to the following equation:

For 0.01–0.2 M Na^+ :

$$T_m = 16.6 \log M + 0.41 (\text{GC}) + 81.5 - 0.72 (\% \text{ formamide})$$

For Na^+ concentrations above 0.4 M:

$$T_m = 81.5 + 0.41 (\text{GC}) - 0.72 (\% \text{ formamide})$$

To obtain a large increase of *in situ* hybridization signal for rDNA, hybridize with rRNA in 80% formamide at 50°–55°C, instead of 70% formamide at 37°C.

Finally, it should be mentioned that during the *in situ* hybridization procedure, relatively large amounts of DNA can be lost (Raap et al., 1986).

Additional hybridization variables

Additional parameters must be considered when calculating the optimal hybridization conditions including the probe length, probe concentration, the inclusion of dextran sulfate, the extent of mismatch between probe and target, the washing conditions, and whether the probes will be single- or double-stranded.

Probe length

The rate of the renaturation of DNA in solution is proportional to the square root of the (single-stranded) fragment length. Consequently, maximal hybridization rates are obtained with long probes. However, short probes are required for *in situ* hybridization because the probe has to diffuse into the dense matrix of cells or chromosomes. The fragment length also influences thermal stability. The following formula, which relates the shortest fragment length in a duplex molecule to change in T_m , has been derived:

Change in $T_m \cdot n = 500$ (n = nucleotides).

Probe concentration

The probe concentration affects the rate at which the first few base pairs are formed (nucleation reaction). The adjacent base pairs are formed afterwards, provided they are in register (zippering). The nucleation reaction is the rate limiting step in hybridization. The kinetics of hybridization is considered to be a second order reaction [$r = k_2$ (DNA) (DNA)]. Therefore, the higher the concentration of the probe, the higher the reannealing rate.

Dextran sulfate

In aqueous solutions dextran sulfate is strongly hydrated. Thus, macromolecules have no access to the hydrating water, which causes an apparent increase in probe concentration and consequently higher hybridization rates.

Base mismatch

Mismatching of base pairs results in reduction of both hybridization rates and thermal stability of the resulting duplexes. To discriminate maximally between closely related DNA sequences, hybridize under fairly stringent conditions (e.g. at $T_m - 15^\circ\text{C}$). On the average, the T_m decreases about 1°C per % (base mismatch) for large probes. Mismatching in oligonucleotides greatly influences hybrid stability; this forms the basis of point mutation detection.

Stringency washes

During hybridization, duplexes form between perfectly matched sequences and between imperfectly matched sequences. The extent to which the latter occurs can be manipulated to some extent by varying the stringency of the hybridization reaction. (See above.)

To remove the background associated with nonspecific hybridization, wash the sample with a dilute solution of salt. The lower the salt concentration and the higher the wash temperature, the more stringent the wash.

In general, greater specificity is obtained when hybridization is performed at a high stringency and washing at similar or lower stringency, rather than hybridizing at low stringency and washing at high stringency.

Use of single-stranded versus double-stranded probes

A number of competing reactions occur during *in situ* hybridization with double-stranded probes. These include:

- ▶ Probe renaturation in solution
- ▶ *In situ* hybridization
- ▶ *In situ* renaturation (possibly, for ds targets)

Consequently, the use of single-stranded probes has advantages for *in situ* hybridization. Such probes can be made by using the single-stranded M13 (or like bacteriophage cloning vectors) as template, or by using transcription vectors which permit the production of large amounts of single-stranded RNA. (See Chapter 4 for a detailed description).

Competition *in situ* hybridization

Recombinant DNA isolated from eukaryotic DNA often contains genomic repetitive sequences (e.g., the Alu sequence in humans). *In situ* hybridization to chromosomes with a probe which contains repetitive DNA usually results in uniform staining. However, unlabeled competitor DNA (usually total genomic DNA) prevents the repetitive probe sequences from annealing to the target, and leads to stronger *in situ* hybridization signals from the unique sequences in the probe. (This approach was first described for *in situ* hybridization by Landegent et al., 1987; Lichter et al., 1988a; Pinkel et al., 1988.) Obviously, the greater the complexity of probe (plasmids < phages < cosmids < yeast artificial chromosomes < chromosome libraries), the greater the need for competition *in situ* hybridization. This approach has proved particularly useful for *in situ* hybridization with DNA isolated from chromosome-specific libraries (CISS-hybridization); a specific chromosome can be fluorescently labeled over its full length (Lichter et al., 1988a,b; Cremer et al., 1988; Pinkel et al., 1988).

Oligonucleotide hybridization

The rules given for hybrid stability and kinetics of hybridization can probably not be extrapolated to hybridization with oligo-deoxynucleotides. For *in situ* hybridization, the advantages of oligonucleotides include their small size (good penetration properties) and their single-strandedness (to prevent probe reannealing, as outlined in Chapter 1).

The small size, however, is also a disadvantage because it covers less target. The nonradioactive label should be positioned at the 3' or the 5' end; internal labeling affects the T_m too much.

In an experiment with 20-mers of 40 – 60% GC content, start with the hybridization conditions described below. Depending on the results obtained, you may decide to use other stringency conditions.

Standard *in situ* hybridization conditions

Department of Cytochemistry and Cytometry, University of Leiden, Netherlands.

For “large” DNA probes (≥ 100 bp):

- 50% deionized formamide
- 2× SSC (see below)
- 50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer; pH 7.0
- 1 mM EDTA
- carrier DNA/RNA (1 mg/ml each)
- probe (approx. 20–200 ng/ml)

Optional components:

- 1× Denhardt’s (see below)
- dextran sulfate, 5–10%
- Temperature: 37°–42°C
- Hybridization time: 5 min–16 h

For synthetic oligonucleotides:

- 25% formamide
- 4× SSC (see below)
- 50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer; pH 7.0
- 1 mM EDTA
- carrier DNA/RNA (1 mg/ml each)
- probe (approx. 20–200 ng/ml)
- 5× Denhardt’s (see below)
- Temperature: room temperature
- Hybridization time: 2–16 h

Composition of SSC and Denhardt’s solution

1× SSC: 150 mM NaCl, 15 mM sodium citrate; pH 7.0:
Make a 20x stock solution (3 M NaCl, 0.3 M sodium citrate).

50× Denhardt’s:
1% polyvinylchloride, 1% pyrrolidone, 2% BSA.

