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Preparation of probes for in situ hybridization

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I. DNA oligonucleotide probes:

- DNA oligonucleotides are the preferable probes at present. They are synthesized chemically, which allows for the incorporation of amino-modified nucleotides (amino-allyl T) at defined positions in the sequence. These free amines are chemically coupled to fluorophores after synthesis. The modified Ts should be about 10 bases apart to prevent quenching and high background. The GC content of the oligo should be around 50%, its length can be variable, but 40-50 bases (5 fluorochromes per molecule of probe) works well in most cases. The signal can be further increased by using several different oligonucleotides against the same RNA.

- After synthesis, the oligo should be purified by either gel electrophoresis or reverse chromatography on C-18 Sep-Pack columns (Waters). Oligos are then conjugated to activated fluorophores as described below.

II. RNA probes:

- RNA probes are synthesized by in vitro transcription with T3, T7 or Sp6 RNA polymerases.

- The fluorescent label can be incorporated during transcription (following manufacturer indications), by using nucleotides that are already coupled to fluorophores. Unfortunately, these nucleotides are incorporated with a low frequency, and the resulting specific activity of these probes is consequently sequence dependent and low, i.e. less than 1 molecule of fluorophore per 100 bases transcribed. Furthermore, these transcription reactions cannot be phenol extracted, since the fluorochrome labelled RNA partitions with the phenolic phase.

- Alternatively, the fluorescent label can be chemically conjugated to the RNA after probe synthesis, and this can result in specific activities as high as 1 fluorophore per 10 bases transcribed. To this end, RNA is synthesized by standard in vitro transcription reactions, except that UTP is replaced by an equal concentration of amino-allyl UTP (Sigma), or by a mixture of UTP/amino-allyl UTP (usually at a 1/1 ratio). Unlike fluorescent nucleotides, amino-allyl nucleotides are incorporated almost as well as unmodified UTP. Transcription reactions are then phenol extracted, RNA is ethanol precipitated, resuspended in 1 x SSC, and unincorporated nucleotides are removed by two rounds of gel filtration (1 x SSC buffered P30 micro-spin column, BioRad). RNA is then again ethanol precipitated and resuspended in water.

  NB: It is very important to design RNA probes such that no polylinker sequences are present in the resulting transcript. The polylinker often contains GC rich stretches that induce cross-hybridization with ribosomal RNA (Witkiewicz H, Bolander ME, Edwards DR, 1993, Biotechniques vol 14, pp 458-463).

III. Chemical conjugation of the amino-modified nucleic acids with activated fluorophores:

- The nucleic acid to be labelled should be resuspended in 70 µl of 0.1 M NaHCO3 buffer, pH 8.8. Different amounts of material will result in different specific activities of the probe. 5-10 µg of nucleic acid will yield a high specific acitity (60-80% of the free amine will react), while 50 µg will yield a lower activity (5-20% of the

  free amine will be conjugated). Labelling is initiated by adding 30 µl of DMSO containing the activated fluorophore. In principle, any amine reactive compound could be used. In practice, CY3 (1 vial of monoreactive labelling kit per reaction, Amersham), works very well for as a red fluorophore, and the

succinimidyl ester of Oregon green 488 (1mg of compound per reaction, Molecular Probes) as a green dye. Labeling is then conducted for 24-48 h in the dark, at room temperature, with occasional vortexing.

NB: Any trace of free amine, such as Tris base, should be removed from the nucleic acid, as it will also react with the fluorophore.

- Unreacted dye is then removed by either two rounds of ethanol precipitation and washing (carrier tRNA can be added if needed), or by gel filtration through G50 columns. Specific activity of the probes is calculated by absorption spectroscopy.

  NB: It is usually desirable to incorporate as many fluorophores per molecule of probe as possible, but it should be noted than more than 4-5 molecules of dye per 40nt probe often results in a high background during in situ hybridization.