cDNA preparation and cDNA library

cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase. In eukaryotes, a poly-(A) tail (consisting of a long sequence of adenine nucleotides) distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription.

mRNA extraction: Firstly, the mRNA is obtained and purified from the rest of the RNAs. Several methods exist for purifying RNA such as Trizol extraction and column purification. Column purification is done by using oligomeric-dT nucleotide coated resins where only the mRNA having the poly-A tail will bind. The rest of the RNAs are eluted out. The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.

cDNA construction: i) Once mRNA is purified, oligo-dT is tagged as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand.

ii) Now, the mRNA is removed by using an RNAse H enzyme or alkali RNA digestion which hydrolyzes RNA but not DNA and leaves a single stranded cDNA (sscDNA).

iii) This sscDNA is converted into a double stranded DNA with the help of DNA polymerase. However, for DNA polymerase to synthesize a complementary strand a free 3'-OH end is needed. This is provided by the sscDNA itself by generating a hair pin loop at the 3' end by coiling on it. The polymerase extends the 3'-OH end and later the loop at 3' end is opened by the scissoring action of S1 nuclease.

Oligo-dC primer can be hybridized to the sscDNA for second strand synthesis. To do this, the 3′ end of each cDNA strand is elongated by adding several residues of a single nucleotide through the action of terminal transferase, a unique DNA polymerase that does not require a template, but simply adds deoxynucleotides to free 3′ ends. A synthetic oligo-dC primer then is hybridized to this 3′ oligo-dG. DNA polymerase, which uses the oligo-dC as a primer, then is used to synthesize a DNA strand complementary to the original cDNA strand. These reactions produce a complete double-stranded DNA molecule corresponding to each of the mRNA molecules in the original preparation. Each double-stranded DNA, also called cDNA, contains an oligo-dC – oligo-dG double-stranded region at one end and an oligo-dT – oligo-dA double-stranded region at the other end.

cDNA library preparation: i) To prepare double-stranded cDNAs for cloning, short restriction-site linkers first are ligated to both ends. These are double-stranded DNA segments, usually ≈10 – 12 bp long, that contain the recognition site for a particular restriction enzyme. Restriction-site linkers are prepared by hybridizing chemically synthesized complementary oligonucleotides. The ligation reaction is carried out by DNA ligase from bacteriophage T4, which can join “blunt-ended” double-stranded DNA molecules lacking sticky ends. Although blunt-end ligation is relatively inefficient, the ligation reaction can be driven to completion by using high concentrations of linkers.

ii) The resulting double-stranded cDNAs, which contain a restriction-site linker at each end, are treated with the restriction enzyme specific for the linker; this generates cDNA molecules with sticky ends at each

end. To prevent digestion of any cDNAs that by chance have a recognition sequence for this restriction enzyme within the cDNA sequence, the mixture of double-stranded cDNAs is treated with the appropriate modification enzyme before addition of the linkers. This enzyme methylates specific bases within the restriction-site sequence, preventing the restriction enzyme from digesting the methylated sites.

iii)The final step in construction of a cDNA library is ligation of the restriction-cleaved double-stranded cDNAs, which now have sticky ends, to plasmid or λ phage vectors that have been cut to generate complementary sticky ends. The recombinant vectors then are plated on a lawn of E. coli cells, producing a library of plasmid or λ clones. Each clone carries a cDNA derived from a single mRNA.