Synthesis of cDNA

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RNA cannot be amplified like DNA. However, it can be converted to copy DNA or cDNA which can be amplified like any other DNA sequence. The process requires "primer" complementary to the target RNA sequence, enzyme called reverse transcriptase, deoxy-nucleotides (dNTPs), magnesium ions and reaction buffer. The end result is formation of cDNA strand that is complementary to the RNA template sequence. The process is also called reverse transcription.

The primer

cDNA may be synthesized by any of the three primers:

1. Gene specific primer
2. Random hexamers
3. Oligo dT primer

Gene specific primers are complementary to the target itself. These can be used in all types of applications but are especially suitable for cDNA synthesis and PCR amplification in the same tube. These are also useful for synthesis of long stretches of cDNA.

Random hexamers are a mixture of all possible combinations of six nucleotide pieces of DNA. These are useful when only small stretches of cDNA or multiple target cDNAs are to be synthesized (Fig 4.1).

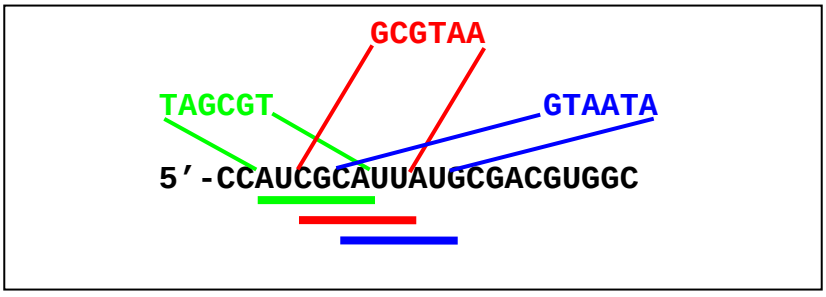

Fig. 4.1 Random hexamers are mixture of all possible combinations of six nucleotides. These anneal randomly to their target RNA and produce relatively short stretches of cDNA.

Oligo dT primers are designed to anneal with the poly-A tail attached at the 3' end of mRNA molecules. Since mRNA constitutes only 1-2% of the total RNA, the yield of

cDNA from oligo dT is low. These are useful for synthesizing long stretches of cDNA or when multiple targets are to be synthesized.

Reverse transcriptase

Reverse transcriptase is an enzyme that can be used for synthesizing DNA copy of RNA. The most commonly used reverse transcriptase is derived from Moloney murine leukaemia virus (MMLV). It is an RNA dependent DNA polymerse that can synthesize DNA complementary to the RNA sequence. In the presence of a primer the DNA synthesis starts from 5' to 3' direction. A thermostable enzyme derived from *Thermus thermophilus (Tth)* has dual activity of reverse trancriptase and DNA polymerase. It can be used for reverse transcription and subsequent PCR in the same reaction tube.

Example

An example of cDNA synthesis from the Human Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA (GenBank NM_002046) is described. The primers were designed by Primer3 plus software (Fig. 4.2).

TGCGTCGCCAGCCGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGTCG**GAGT**
CAACGGATTTGGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAA
AGTGGATATTGTTGCCATCAATGACCCCTTCATTGACCTCAACTACATGGTTTACAT
GTTCCAATATGATTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGG
GAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGC**GAGATCCCTCCAA**
AATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAGTCCACTGGCGTCTTCAC

Fig. 4.2. PCR primers for the human Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), mRNA (GenBank NM_002046.3). The reverse primer (blue) was used as gene specific primer for reverse transcription.

cDNA synthesis

RT Primer (gene specific): 5'-TTGATTTTGGAGGGATCTCG

Reaction conditions:

- 5 X reaction buffer: 4 µl
- 10 mmol dNTPs mix: 2 µl
- RT Primer (10 pmol/µl): 1 µl
- MMLV- Reverse Transcriptase (200 U/µl): 1 µl
- RNase inhibitor (20 U/µl) 1 µl
- Deionized water: 13 µl
- RNA: 2 µl

- Incubation: 42°C for 60 minutes
- RT inactivation: 70°C for 5 minutes

In order to reduce cost the typical reaction volumes may be reduced to suit individual requirements.

PCR amplification

Forward primer: 5'-GAGTCAACGGATTTGGTCGT
(Length: 20, Tm: 60°C, GC 50%)

Reverse primer: 5'-TTGATTTTGGAGGGATCTCG
(Length: 20, Tm: 60°C, GC 45%)

Amplified product size: 238bp

PCR master mix

- PCR buffer with dNTPs: 20 µl
- Forward primer 1 µl (5pmol/µl)
- Reverse primer 1 µl (5 pmol/µl)
- Taq polymerase (5 U/µl) 0.1 µl
- cDNA 3 µl

Thermal cycling:

- Initial denaturation 95°C for 5 minutes.
- Thirty cycles of:
  - Denaturation 95°C for 1 minute
  - Annealing 58°C for 1 minute
  - Extension 72°C for 1 minute 30 seconds
- Final extension 72°C for 3 minutes

Electrophoresis: 6% polyacrylamide at 150 volts for 40 minutes

Staining: 0.1% silver nitrate

Bibliography

1. Lowe K (2001) First strand cDNA synthesis. Oxford Practical Series. Oxfor University Press.
2. Green MR, Sambrook J (2012) Molecular Cloning: A Laboratory Manual. (Fourth Edition). Cold Spring Harbor Laboratory Press.