

# RT-PCR PRINCIPLE AND METHODS

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# ONE-STEP ASSAYS (RT-PCR)

- ⌘ Combine reverse transcription and PCR in a single tube and buffer, using a reverse transcriptase along with a DNA polymerase.
- ⌘ One-step RT-qPCR only utilizes sequence-specific primers.
- ⌘ Adv:
- ⌘ Less experimental variation since both reactions take place in the same tube
- ⌘ Fewer pipetting steps reduces risk of contamination
- ⌘ Suitable for high throughput amplification/screening
- ⌘ Fast and highly reproducible

- ⊠ Disadv
- ⊠ Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions
- ⊠ Detection of fewer targets per sample

# TWO-STEP ASSAYS (RT-PCR)

- ✘ The reverse transcription and PCR steps are performed in separate tubes, with different optimized buffers, reaction conditions, and priming strategies.

Adv:

- ✘ A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions

Disadv:

- ✘ The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination
- ✘ Time consuming
- ✘ Requires more optimization than one-step

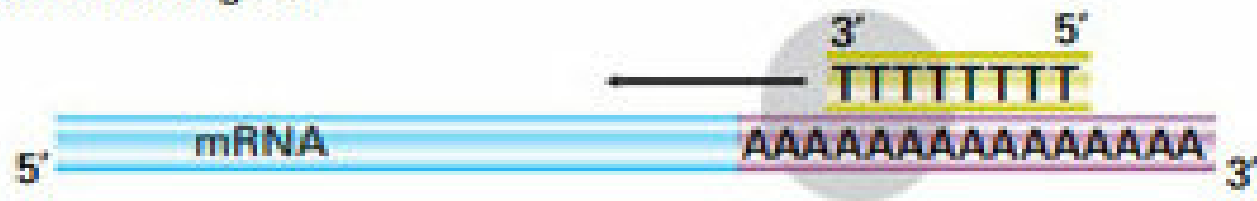
# CHOOSING TOTAL RNA VS. MRNA

- ✘ mRNA may provide slightly more sensitivity
- ✘ mRNA only comprises 1-3% of total RNA sample makes assessing the integrity of mRNA difficult in a total RNA sample
- ✘ total RNA is often used because it has important advantages over mRNA as a starting material.
- ✘ First, fewer purification steps are required, which ensures a more quantitative recovery of the template and a better ability to normalize the results to the starting number of cells.
- ✘ ribosomal RNA (rRNA) makes up more than 80% of total RNA samples, the integrity of the major rRNA species (18S and 28S for mammalian rRNA) is routinely used to assess the RNA sample integrity.

# Primers for Reverse Transcription

- ⊠ Three different approaches can be used for priming cDNA reactions in two-step assays:
  - Oligo(dT) primers,
  - Random primers,
  - Sequence specific primers
- Often, a mixture of oligo (dT)s and random primers is used.
- These primers anneal to the template mRNA strand and provide reverse transcriptase enzymes a starting point for synthesis

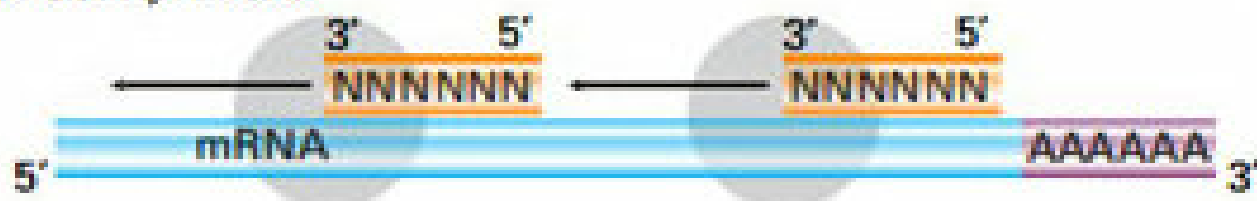
### Standard oligo dT



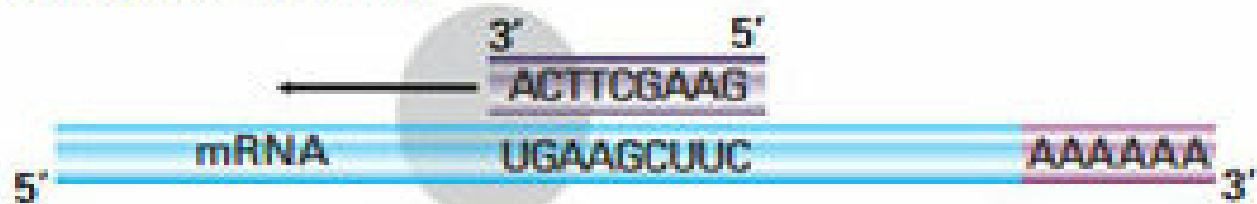
### Anchored oligo dT



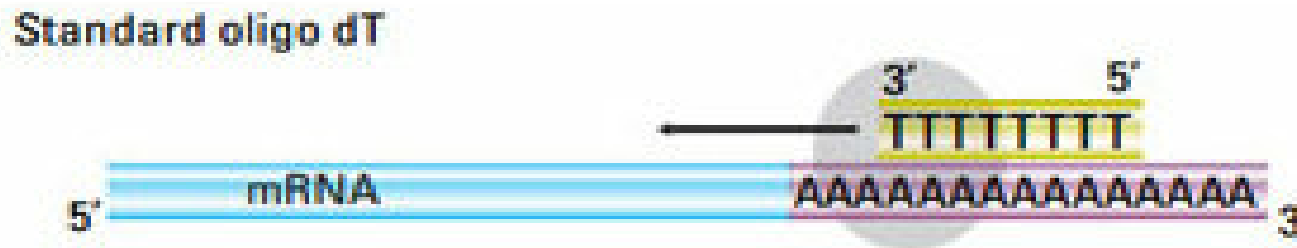
### Random primers



### Gene-specific primers



- ☒ Stretch of thymine residues that anneal to poly(A) tail of mRNA



Adv: Generation of full length cDNA from poly(A)-tailed mRNA

Disadv: Only amplify gene with a poly(A) tail



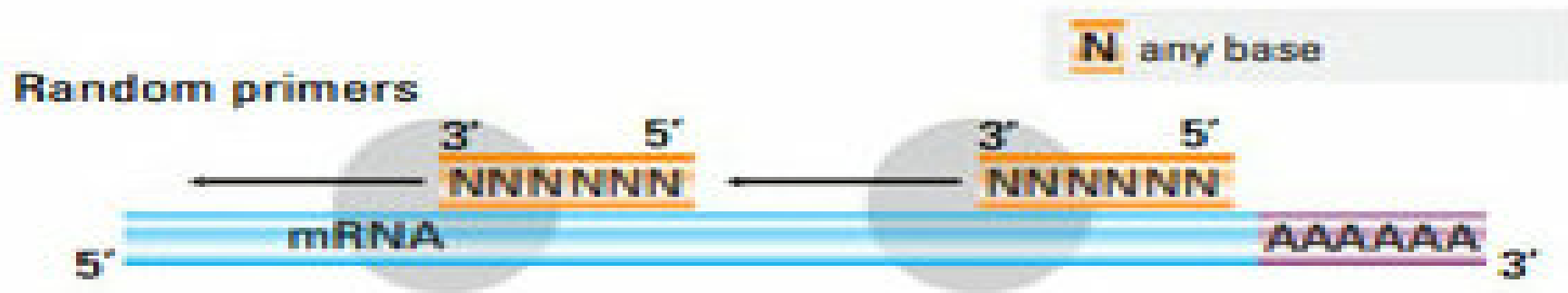
- anchored oligo(dT)s contain one G, C, or A (the anchor) residue at the 3' end



Good to use if little starting material is available

Anchor ensures that the oligo(dT) primer binds at the 5' end of the poly(A) tail of mRNA

- ⊠ Six to nine bases long, they anneal at multiple points along RNA transcript



Adv:

Anneal to all RNA (tRNA, rRNA, and mRNA)

Good to use for transcripts with significant secondary structures, or if little starting material is available; High cDNA yield

Disadv

cDNA is made from all RNAs which is not always desirable and can dilute mRNA signal

- ⌘ Custom made primers that target specific mRNA sequence

#### Gene-specific primers



Adv:

Specific cDNA pool; Increased sensitivity

Use reverse qPCR primer

Disadv:

Synthesis is limited to one gene of interest

# PRIMER DESIGN

- ✘ PCR primers for the qPCR step of RT-qPCR should ideally be designed to span an **exon-exon** junction, with one of the amplification primers potentially spanning the actual exon-intron boundary (Figure 4).
- ✘ This design reduces the risk of false positives from amplification of any contaminating genomic DNA, since the intron-containing genomic DNA sequence would not be amplified.
- ✘ If primers cannot be designed to separate exons or exon-exon boundaries, it is necessary to treat the RNA sample with RNase-free DNase I or dsDNase in order to remove contaminating genomic DNA.

### 1. Primer spans an exon-intron boundary:



### 2. Primers flank an intron:



# CONTROLS FOR RT-QPCR

- ⊠ A minus Reverse Transcription control (-RT control) should be included in all RT-qPCR experiments to test for contaminating DNA (such as genomic DNA or PCR product from a previous run).
- ⊠ Such a control contains all the reaction components except for the reverse transcriptase.
- ⊠ Reverse transcription should not occur in this control, so if PCR amplification is seen, it is most likely derived from contaminating DNA.