What is Real-Time PCR?

• Is the ability to monitor the progress of the PCR as it occurs.

• Data is collected throughout the PCR process rather than at the end of the PCR process.

• The reaction are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles.

- Absolute quantification – using a standard curve of known DNA target numbers (qPCR of a particular gene such as 16S rRNA, IntA, gyrB, etc….

- Relative quantification – normalised to an endogenous control and compared against a calibrator state such as time zero or expression in one of the compared states (qPCR of gene expression- RNA-cDNA)

GENERAL CONSIDERATIONS 1

Need to start with pure nucleic acid

DNA

O.D. 260/280 ratio between 1.6 – 1.8

RNA

O.D. 260/280 ratio between 1.9 – 2.1

Lower ratios mean there is probably still proteins or other impurities present in the sample

GENERAL CONSIDERATIONS 2

Store DNA at - 20 ^{o}C and RNA at - 80^{o}C

Store oligonucleotide primers in aliquots at - 20 ^{o}C avoid repeated freeze thawing.

Always use barrier filter tips throughout whole procedure including DNA extraction.

Set up PCR reactions in separate clean area away from the real time-PCR machine.

Selecting the chemistry

- TaqMan probe-based fluorogenic 5’nuclease chemistry

- SYBR Green I dye chemistry

TaqMan probe-based fluorogenic 5’nuclease chemistry

This chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles.

This method uses 2 principles:

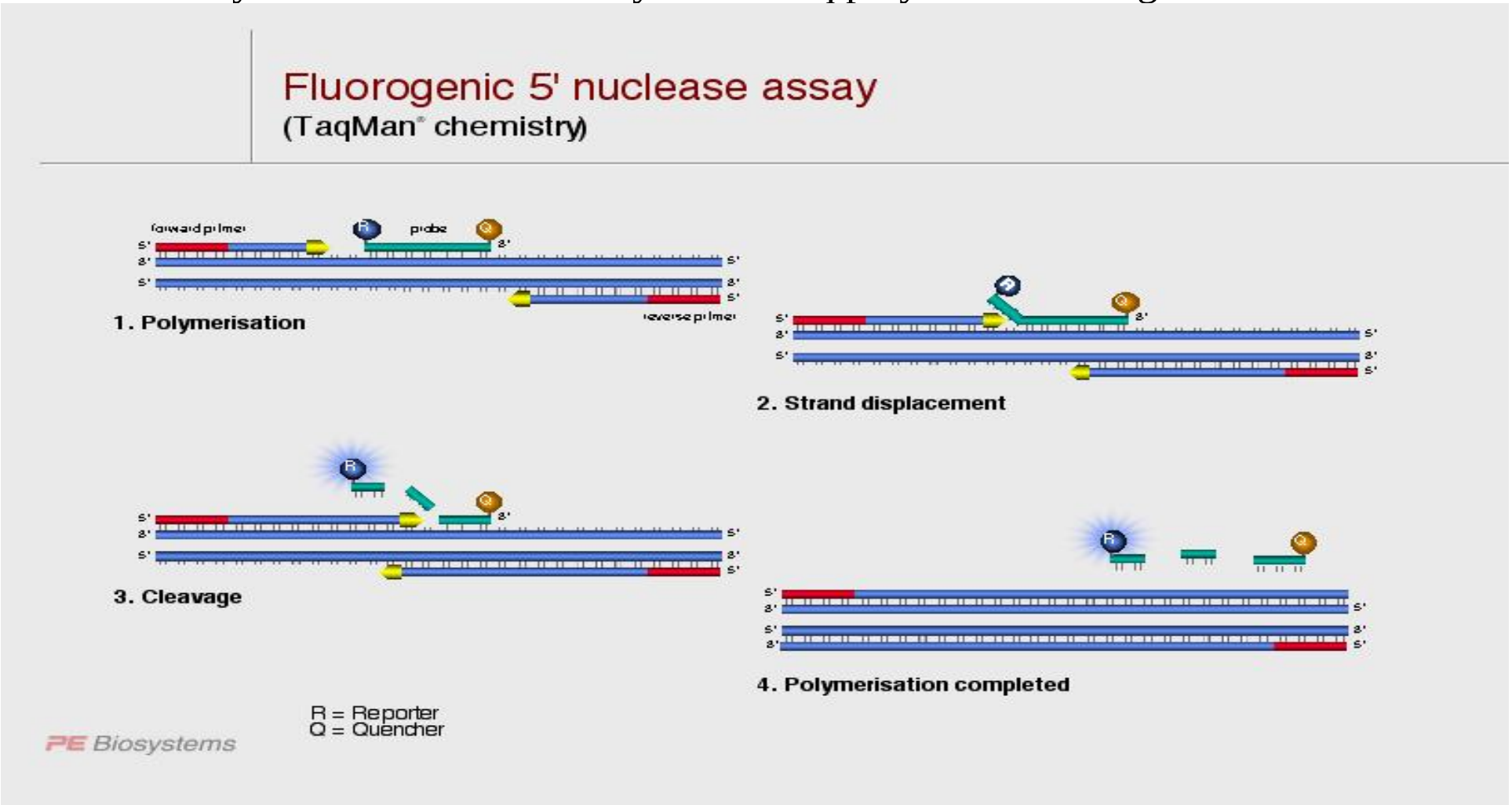
- Fluorescence Resonance Energy Transfer (FRET) technology

- 5’ Nuclease activity of the Taq polymerase

How does it work?

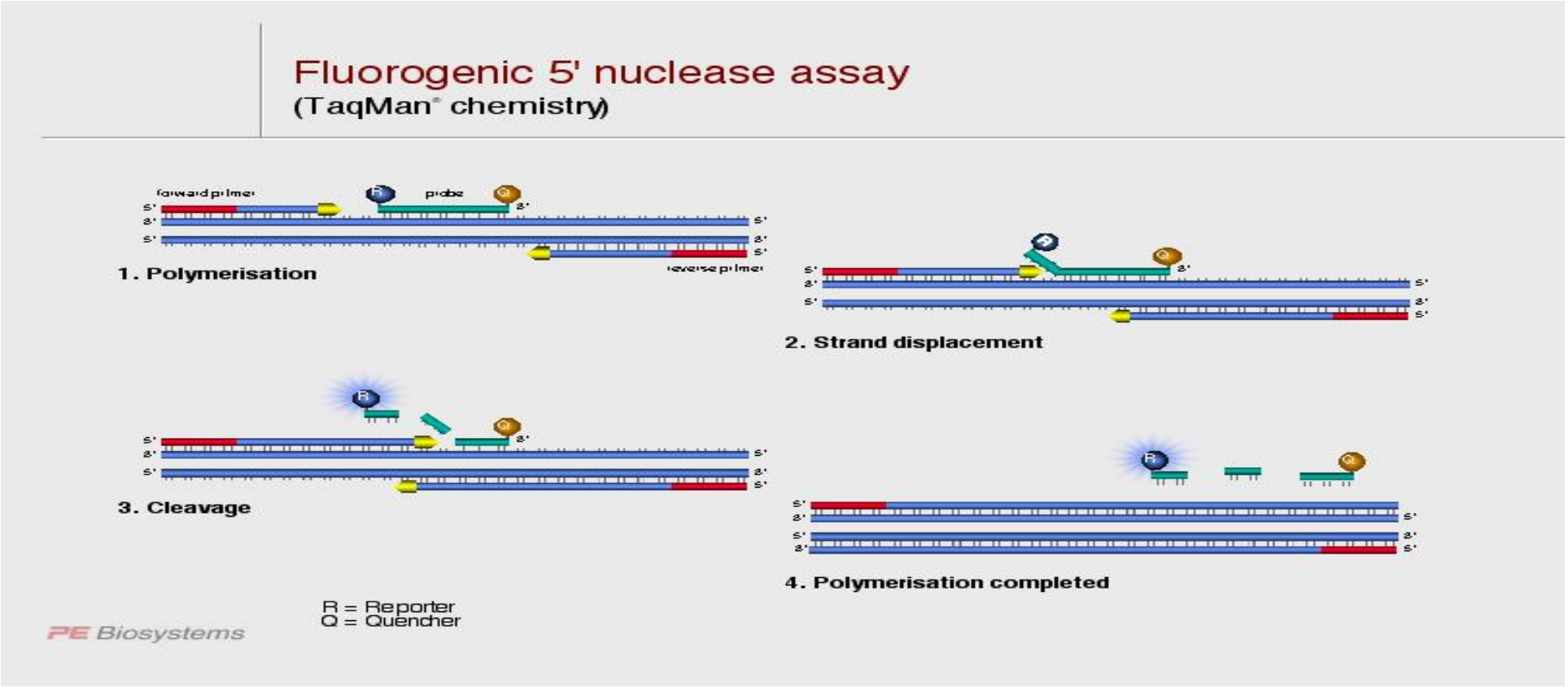
1) An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5’ end (R) and a quencher on the 3’ end (Q)

2) If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5’-nuclease activity of the Taq polymerase during extension



3) This cleavage of the probe:
- Separates the reporter dye from the quencher increasing the reporter dye signal
-Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand.

4) Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of the amplicon produced. The higher the starting copy number of the target, the sooner a significant increase in fluorescence is observed.



SYBR Green dye chemistry

This chemistry uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA forming during PCR.

1) The SYBR Green I dye within the SYBR Green PCR mix immediately binds with all double stranded DNA present in the sample.

2) During the PCR, AmpliTaq Gold DNA Polymerase amplifies the target sequence, which creates the PCR product or amplicon.

3) The SYBR Green I dye the binds to each new copy of double-stranded DNA

4) As the PCR progress, more amplicon is created. The increase in fluorescence intensity is proportional to the amount of double-stranded PCR product produced.

SYBR Green

– Binds to minor groove of double stranded DNA

– Can only be fluoresced when bound

Advantage

– Low cost

Disadvantage

– Binds to all dsDNA in the reaction

Taqman probes

- Specific to target
- Allows for labeling multiple targets

PCR inhibitors

Sample related:

Humic acids, polysaccharides

Extraction related:

SDS, phenol, ethanol, sodium acetate etc..

PCR additives:

DTT, DMSO, Mercaptoethanol, BSA

How much template?

DNA	100pg-1ug
cDNA	10pg-100ng

General guidelines for Taqman probes assays-recommendations

• Primer concentrations: **900nM** (45pmol/50ul reaction = 4.5 ul per reaction of 10 pmol/ul stock)

• Probes concentrations: **250nM**

• This conditions provides high reproducible and sensitive assay

• As a general rule: we should be able to detect and accurate quantify down to less 50 copies for a target sequence

• Amplicon size: 50 to 150 bp

• 30-80% GC content for primers and probes

• T_m probes 10^oC higher than that of the primers

• No G on the 5’ end

• The last five bases on the 3’ of the primers should not contain no more than two C and/or G bases.