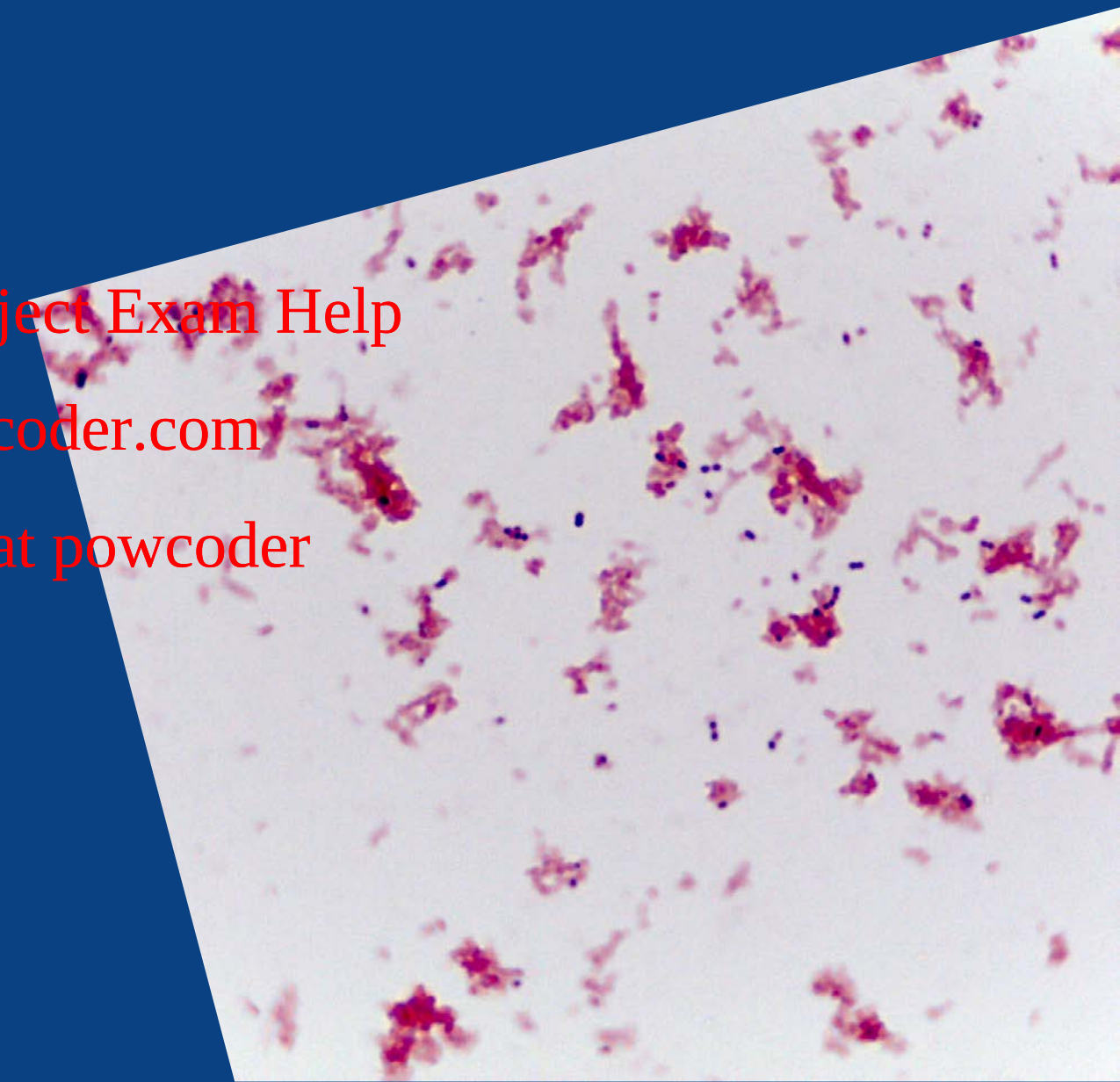
THE UNIVERSITY OF MELBOURNE

Methods of microbiological examination



Helen Billman-Jacobe

Intended Learning outcomes

Be able to describe the principles and give examples of common methods of detecting microbes (or their products) in foods, including:

a. Conventional microbiological methods (cultivation)

b. Immunological methods (ELISA) & enzyme assays (bioluminescence)

c. Molecular detection methods (PCR & qPCR)

Methods of microbiological examination

1. Quantitative – e.g. plate counts (cfu/g)
2. Qualitative – e.g. presence of specific pathogens

- Methods used are standardized, and publically available – and approved/recommended by regulatory agencies

e.g. 'Standard methods for the examination of dairy products'

- We use FSANZ Standard Methods for Food Microbiology.
- also international standards published by the FDA, WHO and ISO (international standards organisation)

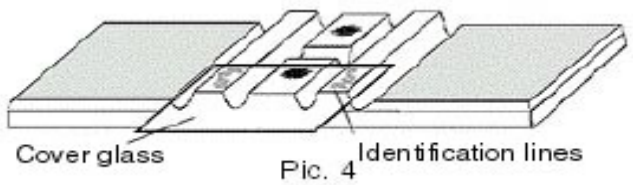
Quantitative tests: Direct Microscopy

- Micro-organisms must be in high concentration – at least 10^6/ml
- Rapid - fast results (minutes)
- Cheap – microscope/person
- Does not distinguish between live and dead cells
- only a small sample volume is viewed, so sample must be representative for the test to give a meaningful result.
- depends on the sample type

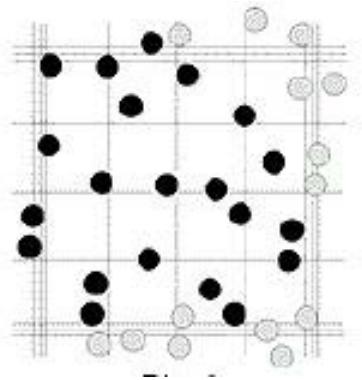
QUS:

- Would these foods be suitable for doing direct counts?
- If so, how would you prepare the sample?
- If not, why not?

Quantitative tests: Direct Microscopy



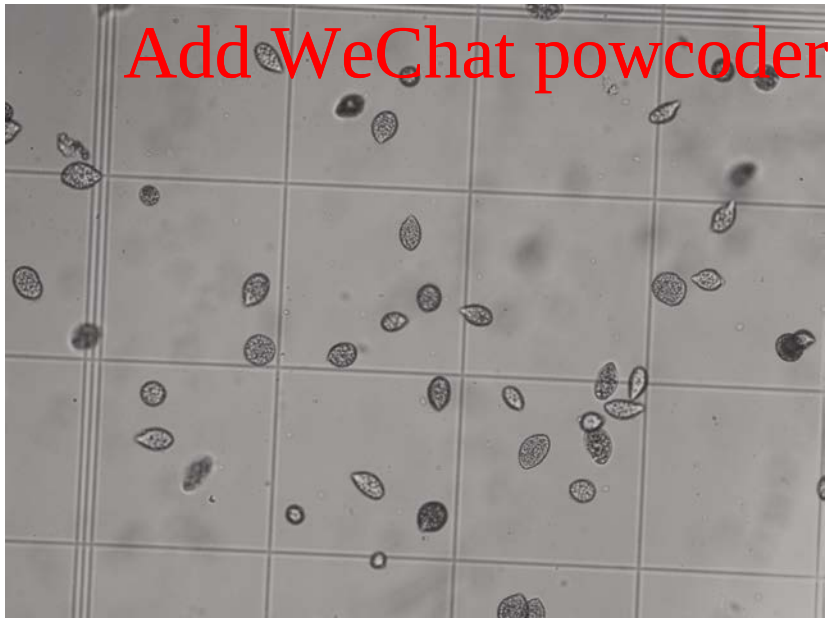
Pic. 4



Bacteria can be counted under the microscope, using a haemocytometer or similar slide. This takes a small, but known volume of fluid, and has a grid etched on the surface of the glass, making counting very easy – as long as there are enough bacteria present!

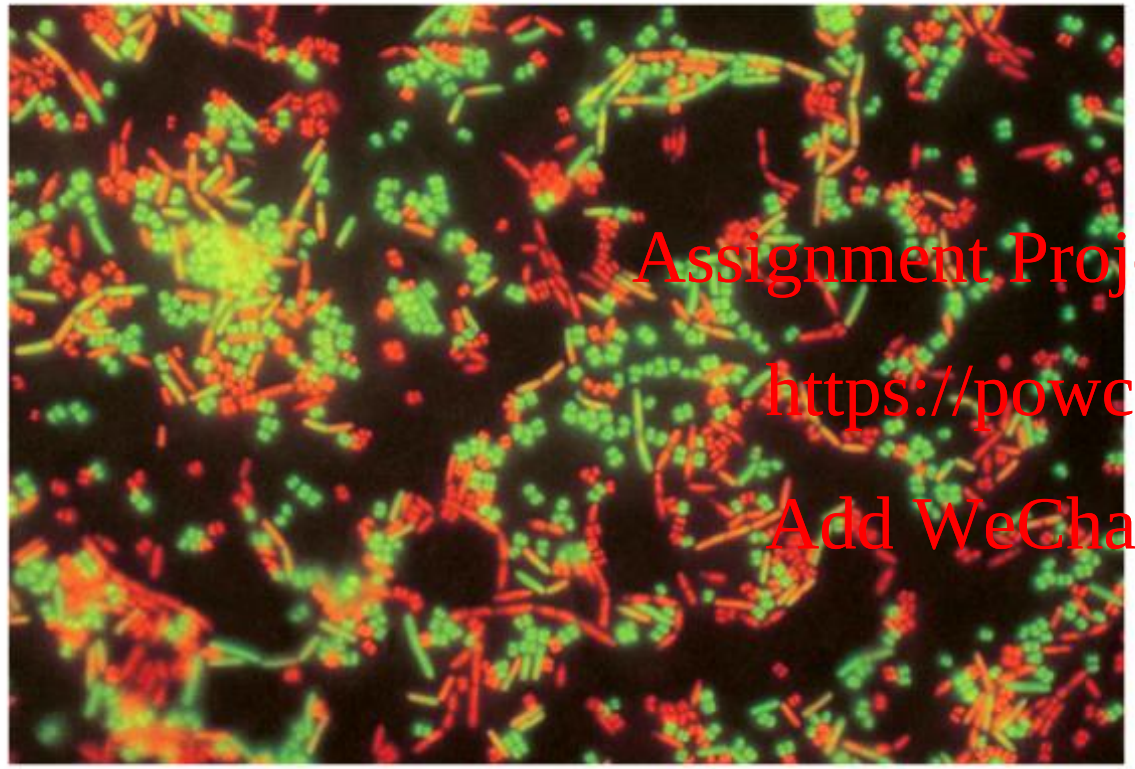
the side of each small square is 50 microns, the depth is 100 microns

Total count/Direct count: reported as cells/ml



Quantitative tests: Direct Microscopy

Many types of light microscopy



Epifluorescent microscopy makes it much easier to pick out the bacteria (bright green) from the background (which is black).

Micrococcus luteus and *Bacillus cereus* stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Cat. No. L7007, L7012). When incubated with the SYTO® 9 stain and the propidium iodide nucleic acid stain provided in this kit, live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red.

LIVE/DEAD *Bac*Light Bacterial Viability Kit *for microscopy

MDS/CSU, 2011

Methods of detection
Enumeration methods using cultivation

- Plate Counts

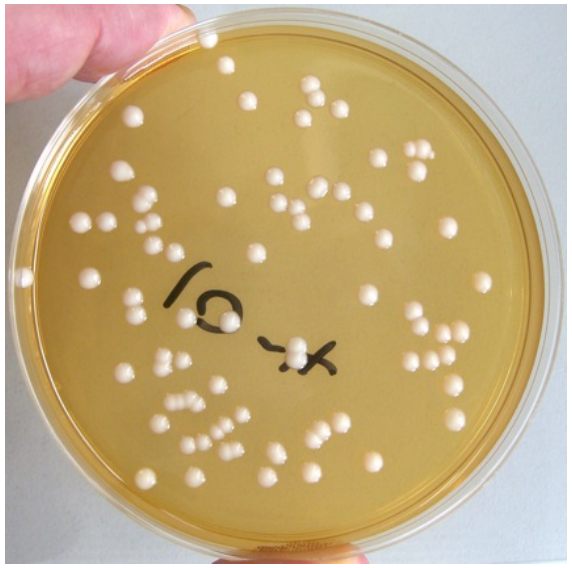


Plate Counts: points to be aware of

- Thermal shock to psychrotrophs (don't shock them!)
- Do NOT dilute with water as this can cause osmotic shock. Use 0.85% NaCl or 0.1% peptone.
- 0.1 ml volume used on standard agar plate
- One colony derived from one or more cells (cfu)
- Test sample is almost certainly has mixed flora.
- Medium type and conditions will select what grows
- Count only plates with 30 – 300 colonies/plate
- More sensitive than direct microscopy but only counts viable cells

Quantitative tests: viable count - types of media

1. **Resuscitation Media**

   For recovery of sub-lethally damaged micro-organisms

2. **Elective/Enrichment Media**

   Encourage the rapid growth of one type of micro-organism e.g. Cooked Meat Medium, Rappaport Vassiliadis broth

3. **Non-Selective Differential Media**

   No selective ingredients. Contains agents capable of distinguishing certain bacteria (e,g. colour change/haemolysis/precipitate)

4. **Selective Differential Media**

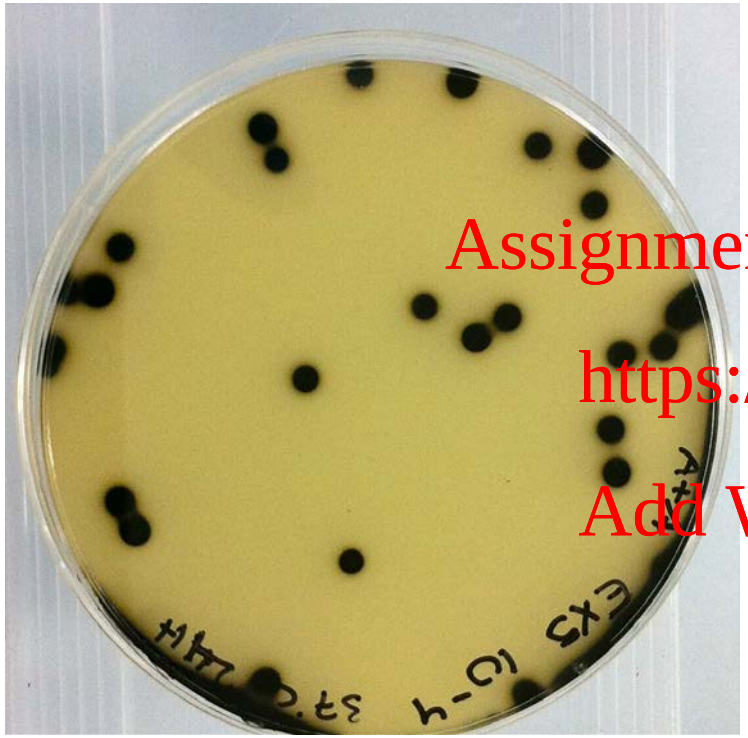
   Contains one or more compounds inhibitory to many micro-organisms but less so to the target organism (selective), plus a differential agent to identify specific bacteria (colour etc.)

Cultivation on selective & differential media

Xylose Lysine Deoxycholate agar

XLD medium for *Salmonella*

Examples of types of cultivation media

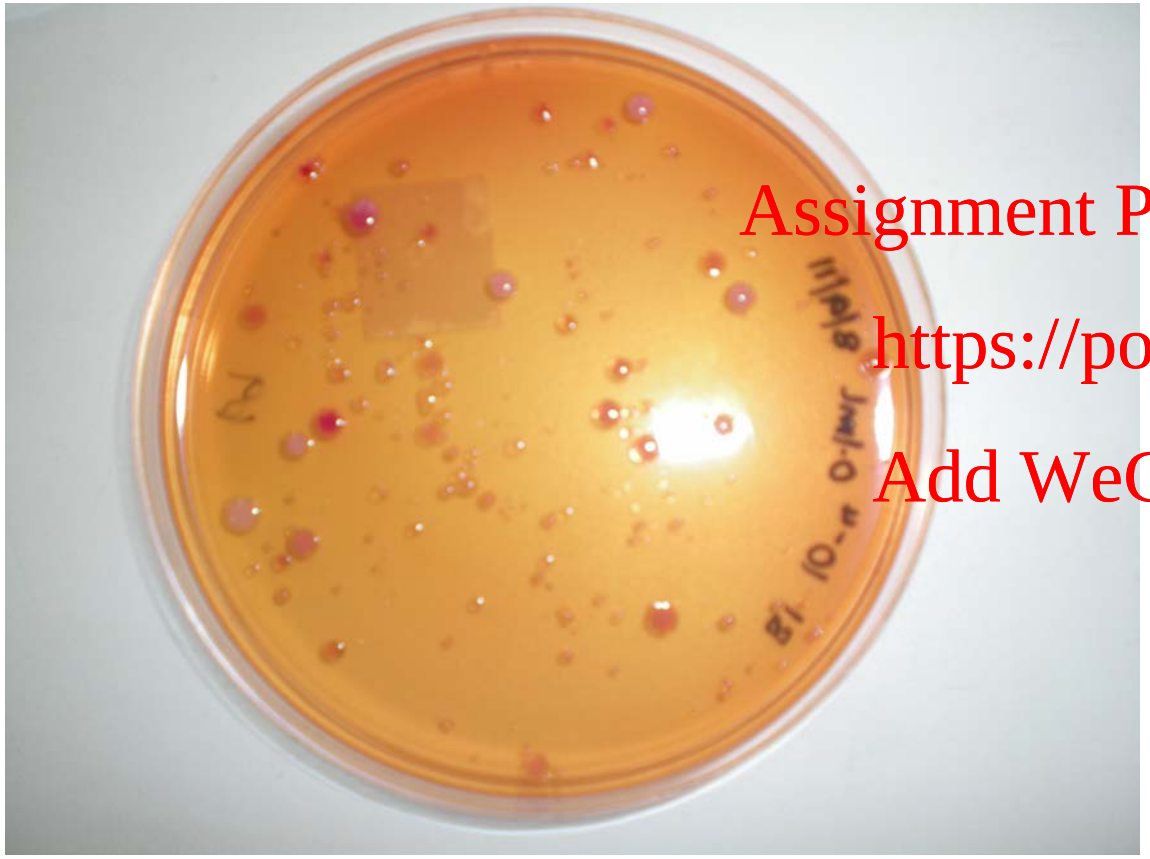


TSC agar with *Clostridium perfringens.*

Tryptose sulfite cycloserine (TSC) agar utilizes the selective inhibitory properties of D-cycloserine and an indicator system involving sulfite and ferric iron

Horse blood agar (HBA) with *S. pyogenes* showing haemolysis

MacConkey agar



MacConkey agar is a selective and differential culture medium for bacteria.

-to selectively isolate Gram-negative and enteric bacteria

-crystal violet and bile salts, inhibits growth of Gram-positive organisms

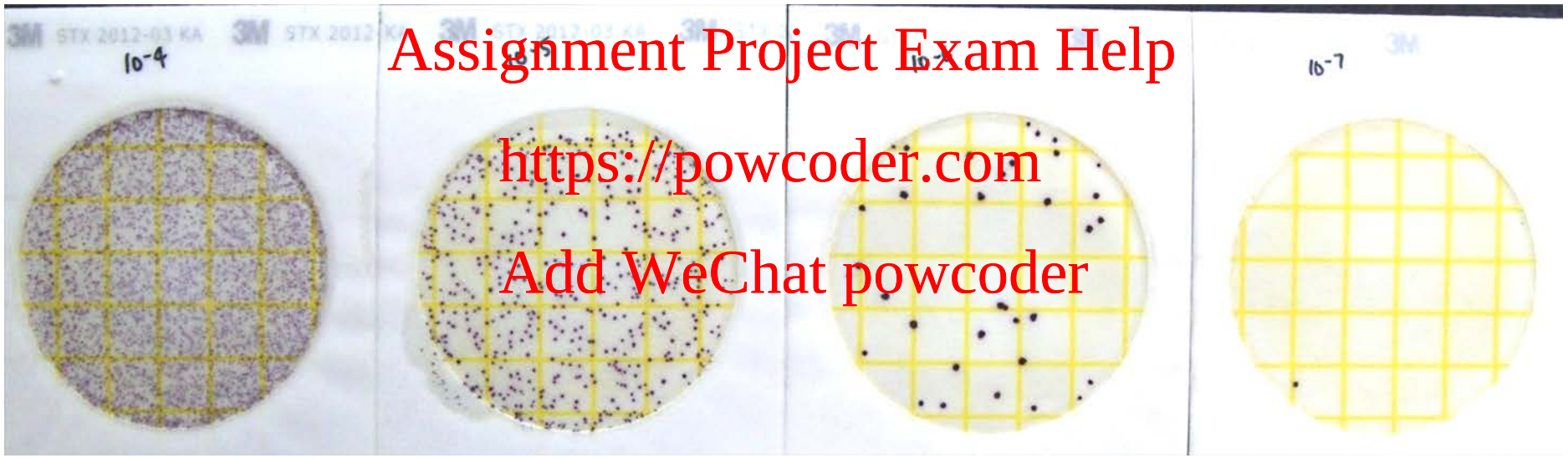
-differentiates them based on lactose fermentation.

Lactose fermenters turn red or pink on MacConkey agar, and nonfermenters do not change color.

-the pH indicator neutral red.

Viable count, *STX*, Petrifilm plates

- 10-fold serial dilutions
- spread (1 mL/plate), in duplicate/triplicate
- incubated 24 hr, 37°C, aerobically



MDS, 2012

Immunological methods: ELISA

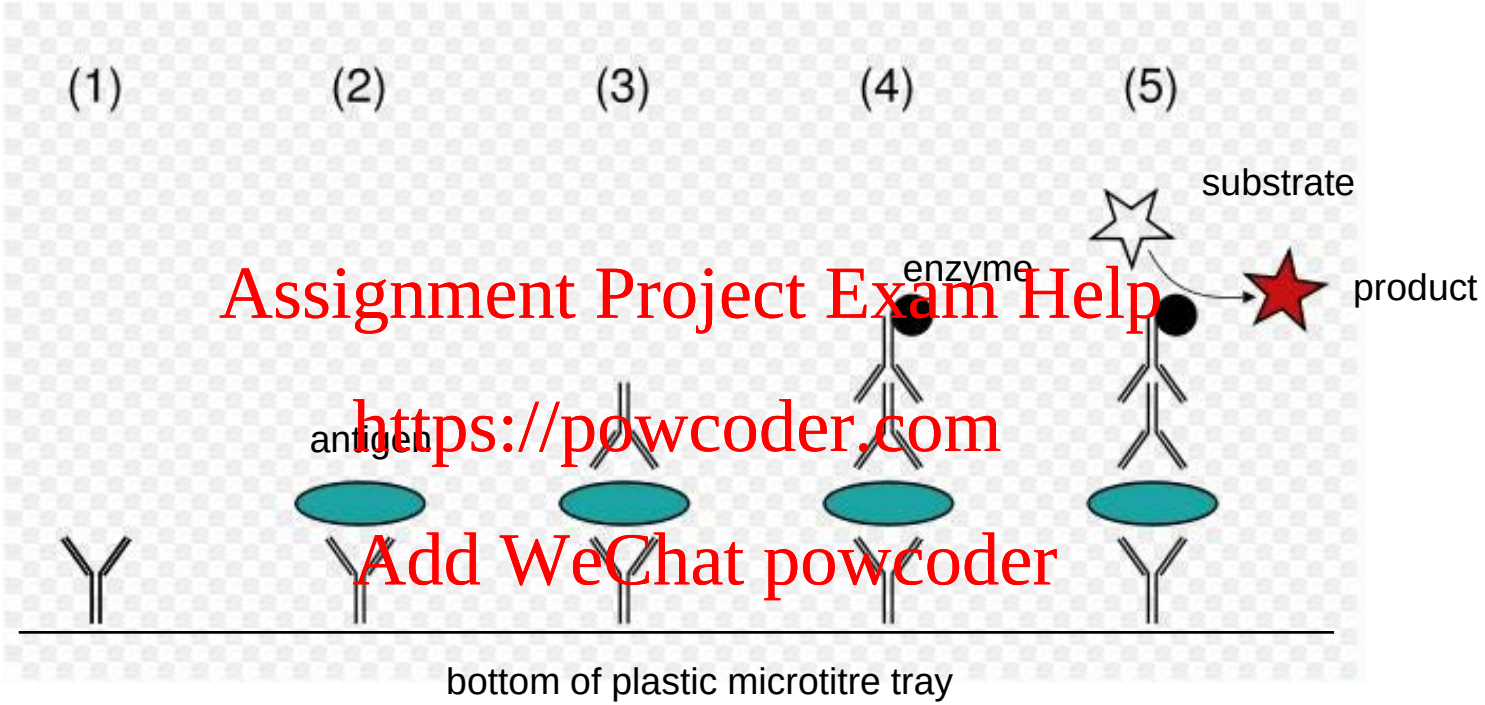
Enzyme Linked ImmunoSorbent Assay (ELISA)

- uses specific antibodies to detect microbes or toxins (or any antigen)
- the antibodies have an enzyme covalently attached
- reaction of the enzyme with a substrate provides signal amplification, increasing sensitivity of test

Limitations:

- Sensitivity varies depending on antibody and type of ELISA being performed, but can need 10^5 – 10^6 organisms for detection. (Therefore pre-enrichment needed = time)
- Specificity of antibodies crucial – cross reactions are possible. They are commercially supplied (usually as a kit)

Method: Direct sandwich ELISA

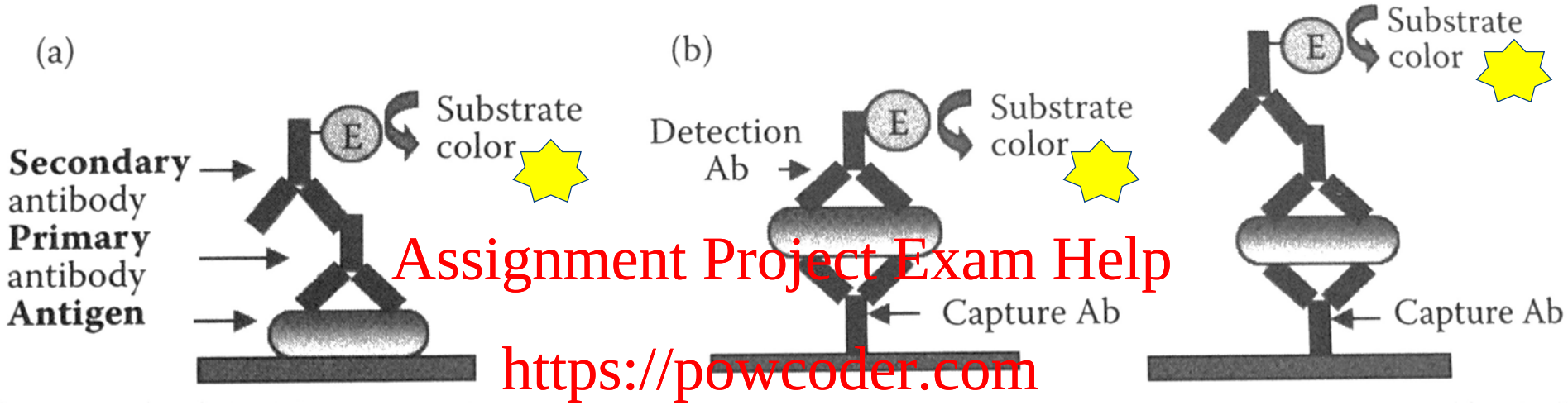


1. Plate coated with capture antibody
2. Sample is added and any antigen present binds to antibody
3. Detecting antibody is added and it binds to antigen
4. An enzyme bound secondary antibody is added which binds to detecting antibody
5. Substrate for enzyme is added
6. Substrate converted by enzyme to give colour change. Intensity of colour is directly proportional to antigen concentration

ELISA

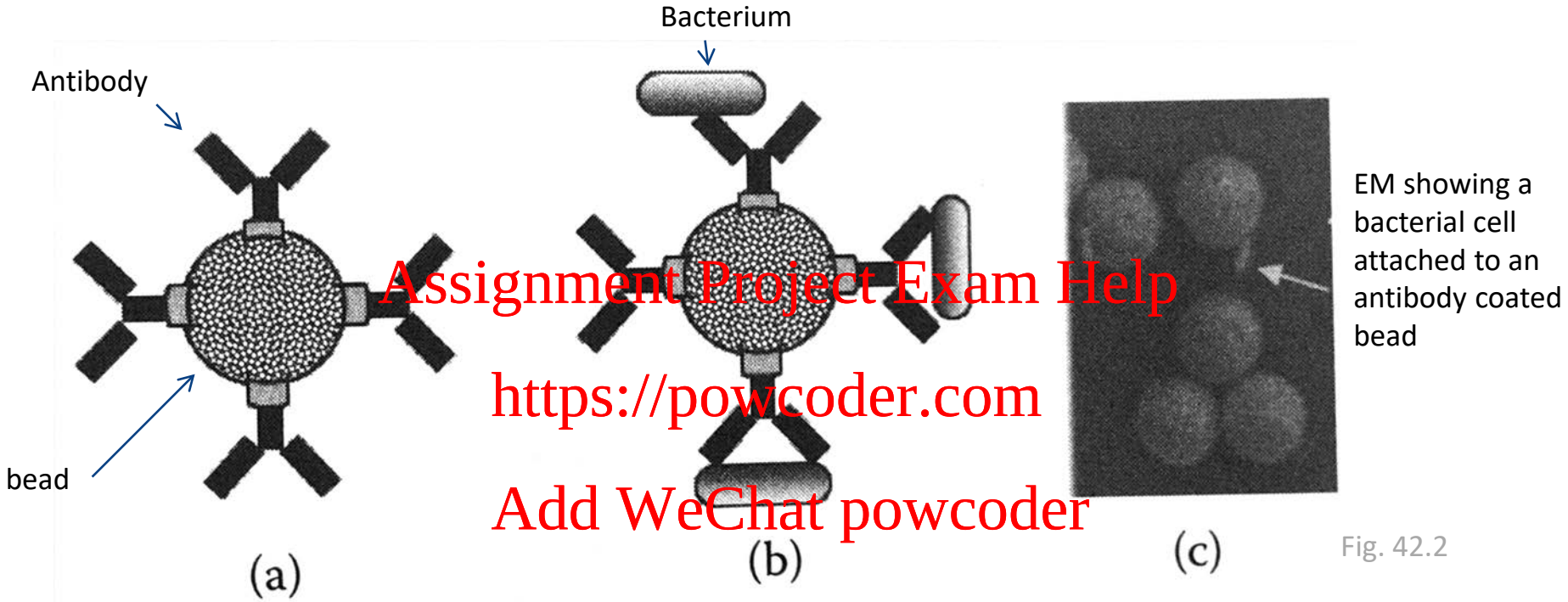
Below is a picture of what an ELISA test looks like. They are usually done in plastic microtitre trays with 96 wells. The positive wells (yellow) are where the antibody-linked enzyme has reacted with the (added) chromogenic substrate, producing a coloured product.

ELISA: types of assay



- Can be used to detect antigen directly bound to plastic well
- or to capture antigen by antibodies bound to well
- Detection can be direct (middle) or indirect (right)

antibody-coated paramagnetic beads



EM showing a bacterial cell attached to an antibody coated bead

Fig. 42.2

Another antibody based test, but here you can mix the beads with food sample, then pull them out with a magnet (paramagnetic means they are only magnetic when in a magnetic field – otherwise they would interact and not distribute in the food sample)

What is the potential advantage of this test over the ELISA?

DNA Sequence based methods

These can be used to quantitate cell numbers (based on the amount of DNA or the number of copies of genes)

•Can also be used to determine the species and strain (i.e. to type strains).

•Can be used to detect presence of virulence genes

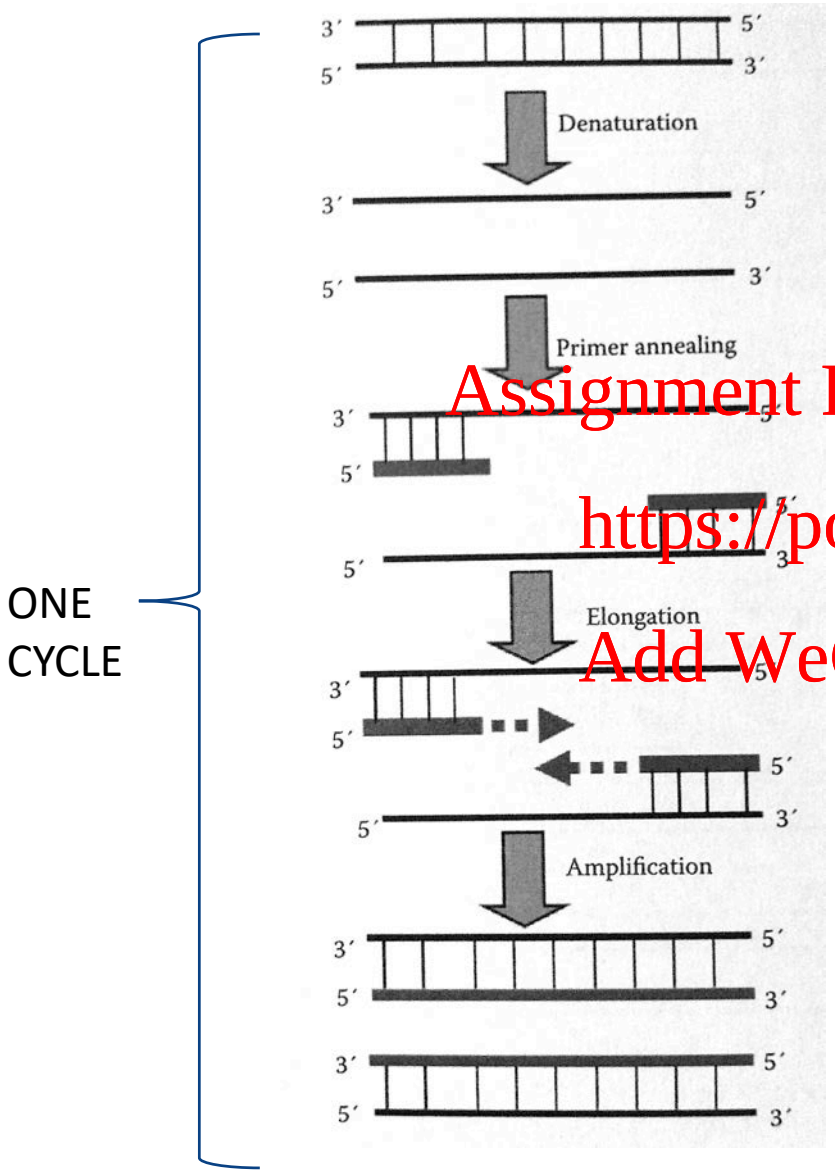
•Can be used to detect the transcription (activity) of genes

e.g.

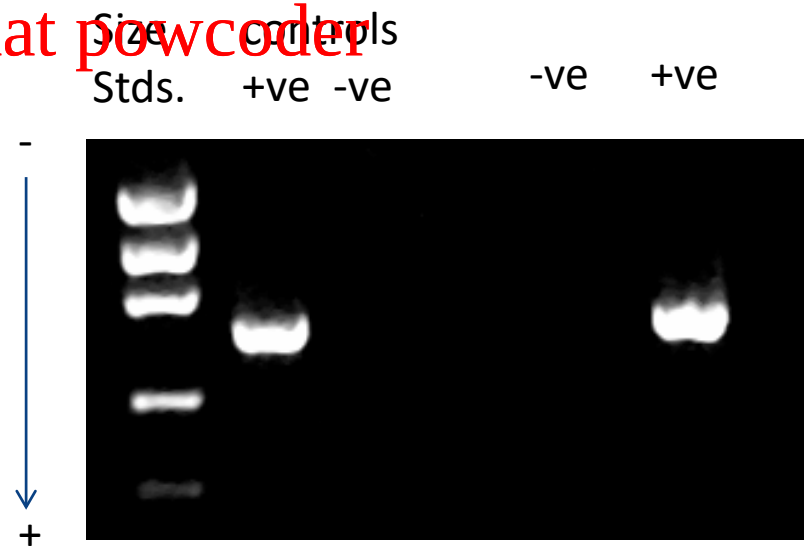
•real-time PCR / RealTime PCR of 16S rRNA genes or mRNA; detects presence and number of particular pathogens or indicator orgs.

•PCR and sequencing of specific genes, such as 16S rRNA, virulence genes (e.g. EHEC)

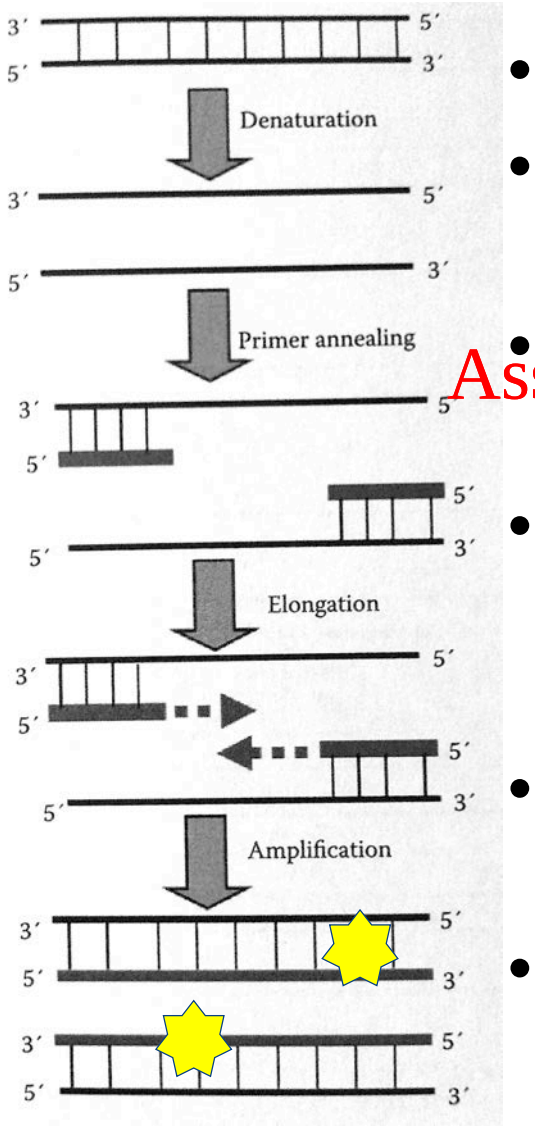
Polymerase Chain Reaction: PCR



- After 30 cycles of PCR
- = ~ 10^9-fold, or 1 billion fold amplification
- detect product by agarose gel electrophoresis

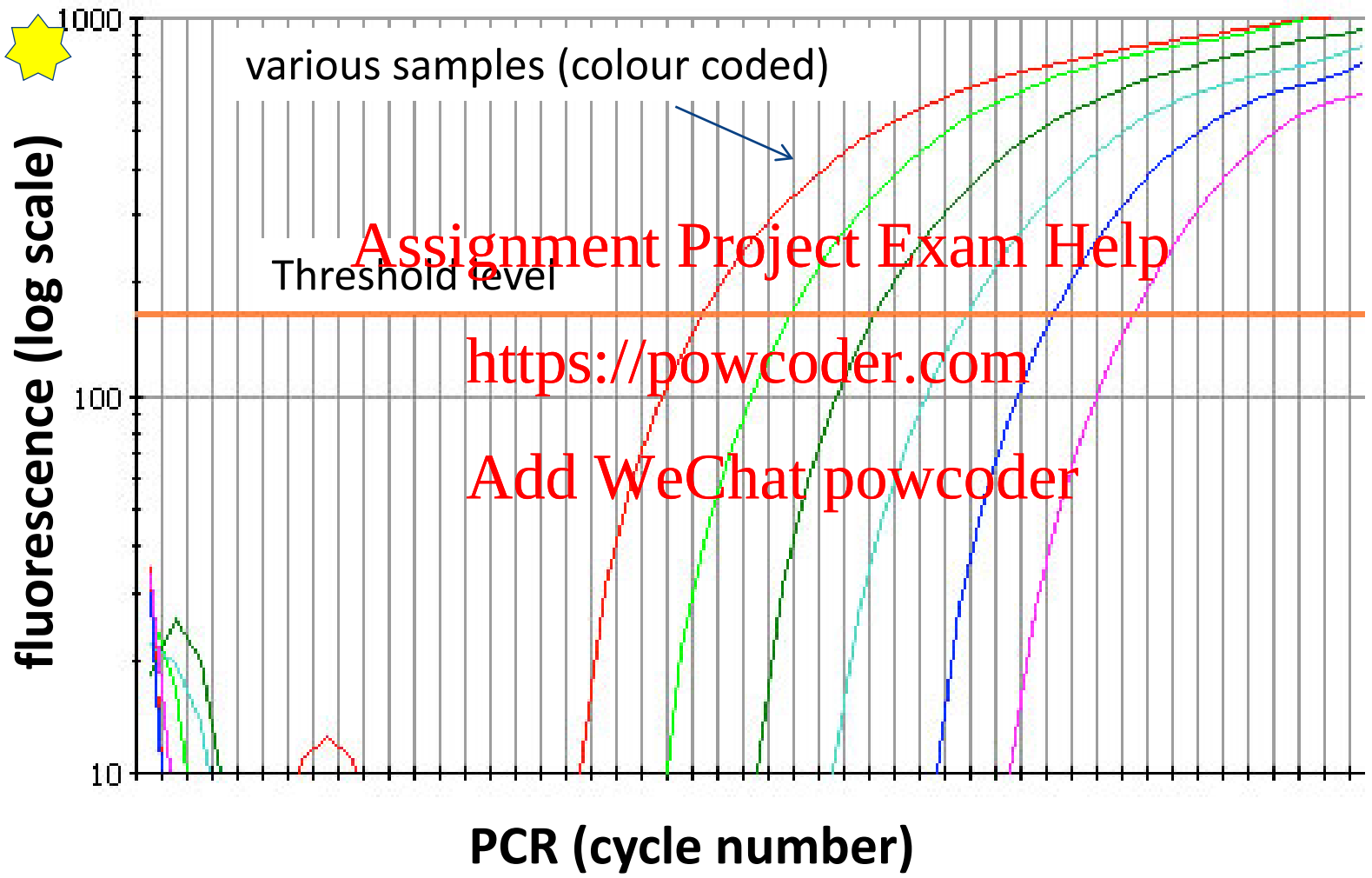


Real-Time or Quantitative PCR



- Normal PCR, but...
- product is detected continuously during PCR, and the amount measured
- detection by a dye that fluoresces only when bound to dsDNA
- in a positive amplification, more dsDNA is produced every cycle, so signal goes up exponentially until reagents run out
- time to +ve depends on initial concentration of target DNA.
- what do you expect the results to look like?

Actual output example (qPCR)



modified from http://www.einstein.yu.edu/uploadedImages/Pathology/Figures/realtime_pcr.jpg?n=3509