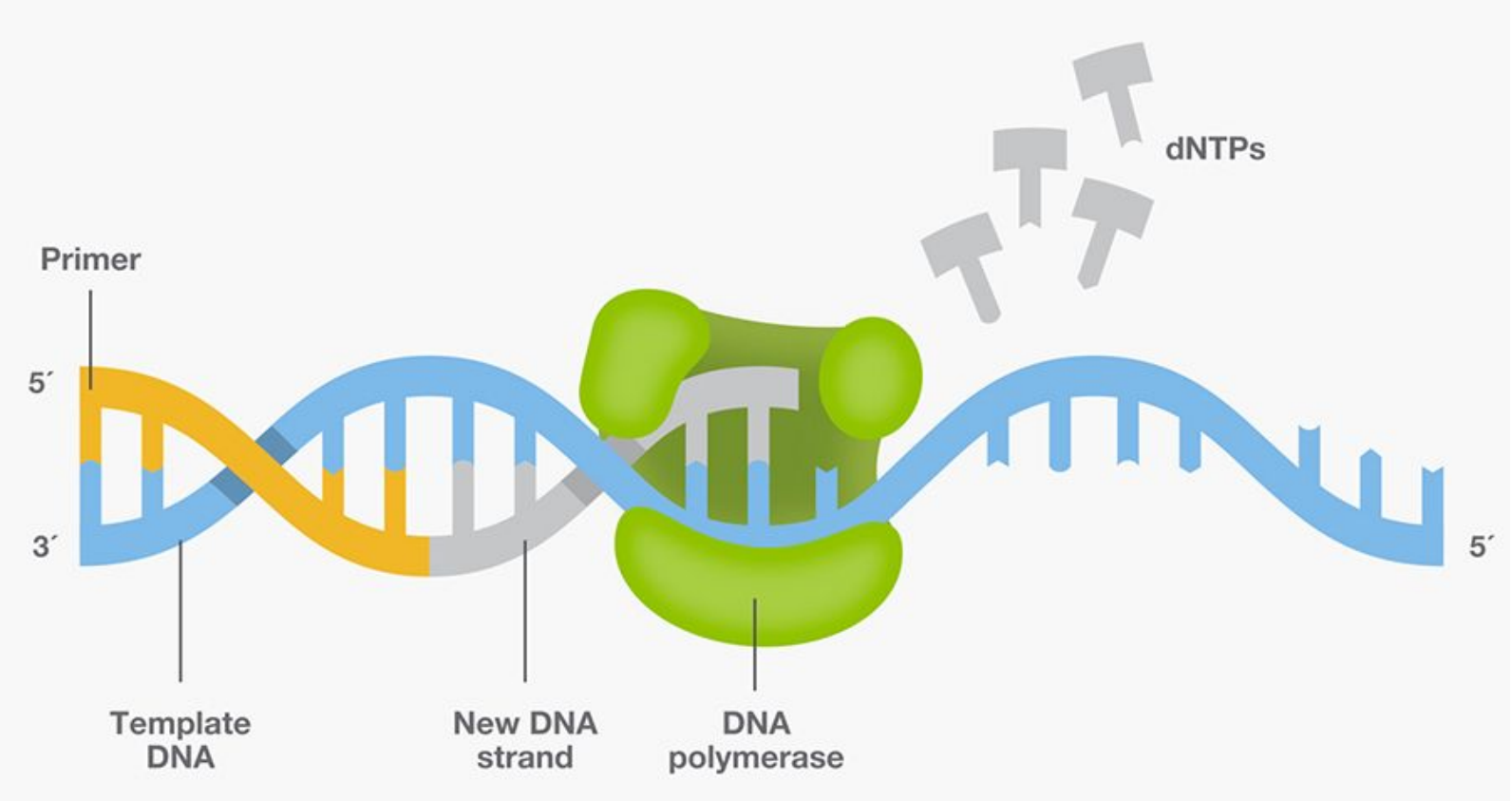
PCR: application in diagnostics



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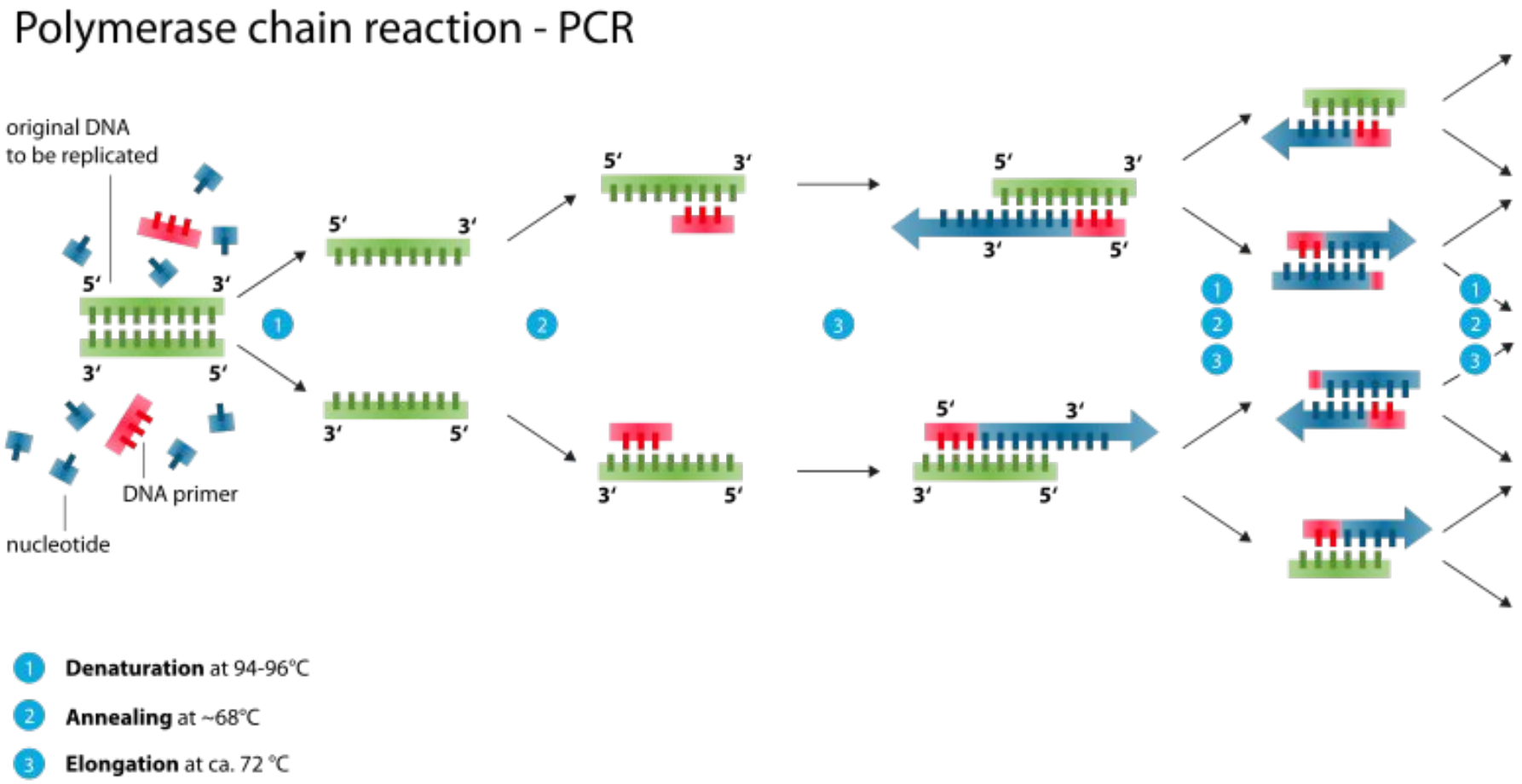
Components and general mechanism

1) Target DNA - contains the sequence to be amplified.
2) Pair of Primers - oligonucleotides that define the sequence to be amplified.
3) dNTPs - deoxynucleotidetriphosphates: DNA building blocks.
4) Thermostable DNA Polymerase - enzyme that catalyzes the reaction
5) Mg^{2+} ions - cofactor of the enzyme
6) Buffer solution – maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme

General mechanism



Application

Diagnostics - The detection of the presence, or absence, of a pathogen and its subsequent identification and characterization.

Features:

Approach 1-> designs primers that are complementary to a DNA target that is specific for the microbe being assayed. For instance, by selecting unique regions of the *Whipple bacillus*' 16S rRNA gene, one can create primers that will amplify only the 16S rRNA gene from the *Whipple bacillus, Tropheryma whippelii.*

Approach 2-> multiplexing, in which multiple specific PCR assays are run simultaneously in the same reaction tube to test for multiple different DNA templates. In multiplex PCR, several sets of primers are added to the reaction in order to generate several different PCR products. For instance, one could have a PCR assay designed to detect bacterial DNA that uses five different specific PCR reactions in one tube, with primer pairs directed toward *S. pneumoniae, N. meningitidis, H. influenzae, Listeria monocytogenes*, and the group *B Streptococcus.*

Advantages/disadvantages

Advantages- High sensitivity and specificity (specific primer design), rapid test, ease of use, and robustness, capability to detect pathogens which are impossible to cultivate on media.

Disadvantages - Requirement of special conditions, high cost equipment, expensive reagents.

PCR in diagnostics

Assays are available for a variety of pathogens, including HIV, HSV, hepatitis B virus, hepatitis C virus, cytomegalo virus, ennterovirus, *Chlamydia trachomatis*, *M. tuberculosis*, *T. whippelii*, and *Neisseria gonorrhoeae*, *Brucella sp*. For the detection of RNA Viruses is applied RT-PCR method (Reverse Transcriptase PCR). Reverse transcriptase is enzyme capable to synthesize DNA strand from RNA template.

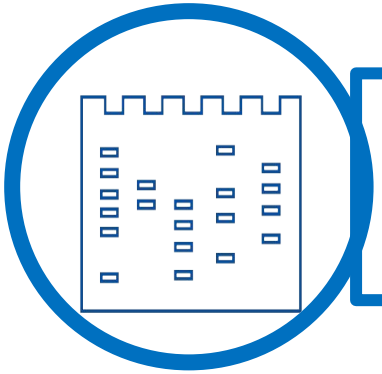
Generally the principle of detection is based on the detection of pathogen's specific DNA/RNA region, amplification of that sequence and analyzing the presence or absence of detection amplicons on electrophoretic agarose gel)

The detection of *Brucella* sp. and strains (cause of brucellosis) using a PCR assay

Procedure

Isolation of DNA

PCR



Gel electrophoresis

Analysis of results

Isolation of DNA

Buffers and reagents

1. Buffer #1 (20mM NaCl, 20mM EDTA, 20 mM Tris-HCl pH= 7,5, 0,5%Triton)
2. Buffer #2 (10mM NaCl, 50mM EDTA, 50mM Tris-HCl pH= 7,5, 1% SDS)
3. 20X SSC (NaCl 175.3g, Sodium Citrate 88,2 g, Dissolve in 800 mL of water, Adjust pH to 7.2 by adding a few drops of concentrated HCl, then adjust volume to 1000 mL). Dilute to use in protocol as 1X SSC.
4. Proteinase K (20 mg/ml)
5. Biophenol (phenol-chloroform- isoamylalcohol)
6. Ammonium acetate 7.5M
7. Absolute ethanol/ 70% Ethanol

Isolation of DNA

Procedure

1. 500 uL of sample (milk, culture, organs tissues, blood etc)
2. Add 500 uL of buffer #1
3. Leave on ice for 30 min
4. Centrifuge at 12000 rpm for 15 min at room temperature or 4 °C
5. Discard the supernatant
6. Add 500 µL of 1x of SSC
7. Vortex
8. Centrifuge at 12000 rpm for 15 min
9. Discard the supernatant
10. Add 500 µL of Buffer #2
11. Mix and vortex
12. Add 20 µL of proteinase K (20mg/ml)
13. Incubate for 2 hours at 50C (or overnight)

Isolation of DNA

14. Add 500 µL of biophenol
15. Vortex
16. Centrifuge at 12000 rpm for 10 min
17. Keep the supernatant in one clean tube
18. Add 1/3 of volume (supernatant) 7.5M ammonium acetate
19. Add 2 volumes of absolute ethanol and mix
20. Centrifuge at 12000 rpm for 10 min
21. Discard the supernatant
22. Add 200 µL of 70% ethanol to wash DNA pellet
23. Centrifuge at 12000 rpm for 10 min
24. Dry pellet at room temperature
25. Dissolved the pellet in 50 µL of HLPC water
26. Store at - 20 °C

PCR

Master mix (x 1)

1. 2.5 µL 10 x PCR buffer
2. 0.5 µL dNTPs (10 mM)
3. 0.5 µL each of: ISP1 and ISP2 (20 pmol/µL) primers for *Brucella* group specific, IS711 and AB (20 pmol/µL) primers for *B. abortus*, IS711 and SV (20 pmol/µL) primers for *B. suis*, IS711 and OV (20 pmol/µL) primers for *B. ovis*, IS711 and BM (20 pmol/µL) primers for *B. melitensis*, ERI1 and ERI2 (20pmol/µL) primers for *B. abortus* vaccine strain S19, IS711 and RB51 (20 pmol/µL) primers for *B. abortus* vaccine strain RB51
4. 17.75 µL H_2O
5. 0.25 µL or 2U Taq polymerase (e.g. Promega Taq)
6. 3 µL of isolated DNA

**Reference Material - positive (DNA of certain strain isolated from pure culture) and negative controls (no DNA)*

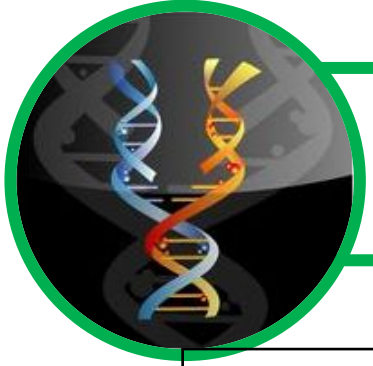
PCR

Composition of PCR Buffer 10x

Tris Cl, pH 8.6	0.5M
KCl	0.5M
MgCl2	015mM
Tween 20	1%
H2O	

PCR → Primer sets

Type	Primer set	Amplicon size
Brucella group specific	ISP1 + ISP2	600 bp
B. abortus	IS711 + AB	498 bp
B. suis	IS711 + SV	285 bp
B. ovis	IS711 + OV	976 bp
B. melitensis	IS711 + BM	731 bp
B. abortus vaccine strain S19	ERI1 + ERI2	178 bp
B. abortus vaccine strain RB51	IS711 + RB51	364 bp



PCR → Primer sets sequences

ISP1	5'- GGT TGT TAA AGG AGA ACA GC -3'
ISP2	5'- GAC GAT AGC GTT TCA ACT TG -3'
IS711	5'- TGC CGA TCA CTT AAG GGC CTT CAT -3'
AB	5'- GAC GAA CGG AAT TTT TCC AAT CCC -3'
SV	5'- GCG CGG TTT TCT GAA GGT TCA GG -3'
OV	5'- CGG GTT CTG GCA CCA TCG TCG -3'
BM	5'- AAA TCG CGT CCT TGC TGG TCT GA -3'
ERI1	5'- GCG CCG CGA AGA ACT TAT CAA -3'
ERI2	5'- CGC CAT GTT AGC GGC GGT GA -3'
RB51	5'- CCC CGG AAG ATA TGC TTC GAT CC -3'

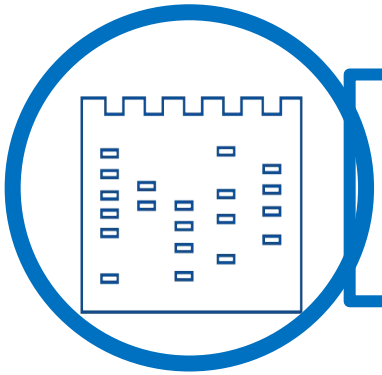
PCR → Regime

PCR amplification for Brucella group specific

Denaturation	95°C	35 s
Annealing	56°C	45 s
Extension	72°C	45 s
Number of cycles	30	

PCR amplification for Brucella spp. and strains

Initial denaturation	94°C	2 min
Denaturation	95°C	20 s
Annealing	55.5°C	20 s
Extension	72°C	30 s
Number of cycles	35	



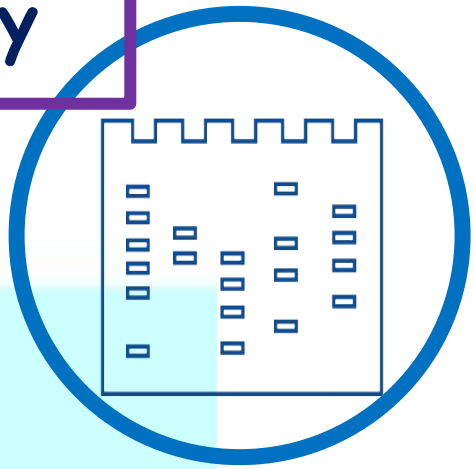
Gel electrophoresis

1. A 1,5% agarose gel stained with ethidium bromide is used
2. 10 µl of the product is loaded with 2 µl loading buffer
3. 2 µl of a 100 bp DNA molecular weight marker is loaded with 2 µl loading buffer a single outside well
4. Gel electrophoresis is performed at 100 to 120V for 30 min

*The composition of LOADING buffer was not mentioned in manual, but on practice it is possible to use loaders like bromphenol blue and xylene cyanol, or cresol red.

Analysis of results

Visually



The positive control should yield fragment sizes of :

600 bp for *Brucella* group specific

498 bp for *B. abortus*

285 bp for *B. suis*

976 bp for *B. ovis*

731 bp for *B. melitensis*

178 bp for *B. abortus* vaccine strain S19

364 bp for *B. abortus* vaccine strain RB51

The negative control should not show any amplicons.

Thanks for attention!