# MBPCR199 Hi-PCR® A1A2 Probe PCR Kit

## Description

Milk is a wholesome diet that most people of every class drink. It is a rich source of calcium and protein. Several elements are also found in the milk like lactose, fat, other vitamins and minerals. Casein is the largest group of proteins in milk, making up about 80% of total protein content. There are several types of casein in milk. β-casein is the second most prevalent and exists in at least 13 different forms. The two most common forms are, A1 β-casein and A2 β-casein. Regular milk contains both A1 and A2 β-casein, but A2 milk contains only A2 β-casein. A1 is considered a genetic mutation that results in the production of the compound BCM7 assumed to be causing the development of unwanted health conditions and illnesses among consumers. Growing number of studies have enumerated the benefits of A2 milk, and the health risks associated with continuous consumption of A1 milk. Nucleic acid amplification-based assays or Polymerase Chain Reaction (PCR) is an alternative method of A1 and / or A2 Genotyping that allows for sensitive and specific detection of A1 and / or A2 DNA from milk samples. Real-Time PCR technique is considerably simple and fast with respect to the standard PCR technique.

**NOTE**: HiMedia's Hi-PCR® A1A2 Probe PCR Kit is for *in-vitro* use only.

## Intended Use

Recommended for sensitive and specific detection and differentiation of A1 and / or A2 in milk samples.

## Principle

Real-time polymerase chain reaction, also called quantitative Polymerase Chain Reaction (qPCR) or kinetic Polymerase Chain Reaction, is a laboratory technique based on the principle of PCR. This technique is used to amplify a targeted DNA sequence by use of hydrolysis probes that are short oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. HiMedia's Hi-PCR® A1A2 Probe PCR Kit is designed to detect the **A2 and A1 alleles in FAM and JOE channels** respectively with **Internal Control in Texas Red channel** in a single tube reaction.

## Positive control

This is a control reaction using a known template (target pathogen). A positive control is usually used to check that the primers have been designed properly and the PCR conditions have been set up correctly.

## Negative Control

A Negative control is needed to ensure that the reagents, equipment, and environment used in the assay is not contaminated. In this reaction, Nuclease free water is used as the template. It is recommended to have minimum 1 reaction of negative control per run.

## Internal Control

This is a control sequence which is amplified in the same reaction tube along with the target sequence (target species) but detected with a different primer (i.e. Multiplex PCR). An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified.



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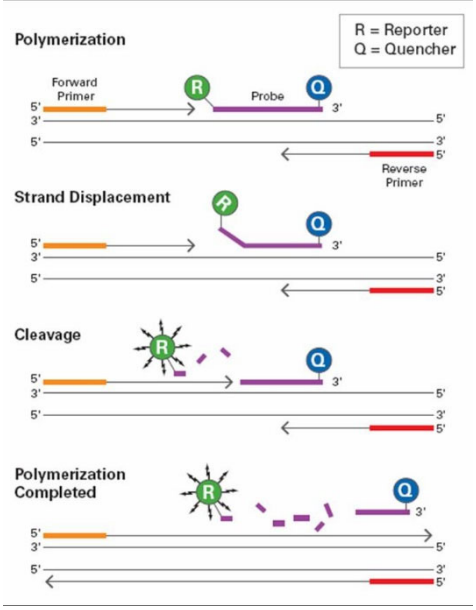
**Diagrammatic representation of preferential binding of probe specific to DNA fragments in Real-time PCR**




**Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' end of the probe respectively**

**Strand displacement:When the probe is intact, the report dye emission is quenched.**

**Cleavage: During each extension cycle,the DNA polymerae cleaves the reporter dye from the probe**

**Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence**

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). The probes are designed such that they anneal within a DNA region amplified by a specific set of primers. During PCR amplification, these probes will hybridize to the target sequences located in the amplicon i.e. the DNA. As the *Taq*DNA polymerase replicates the template with the bound probe, the 5'- nuclease activity of the polymerase enzyme cleaves the fluorescent probe. The end result in cleavage of the probe is separation of the reporter dye from the quencher dye and increasing the reporter dye signal. As the probe is removed from the target strand, primer extension continues to the end of the template strand. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Thus, inclusion of the probe does not inhibit the overall PCR process.

**Features**

- Fast and simple
- Good sensitivity and specific results
- Guaranteed reproducible results

**Sample Source:** Meat / Tissue / Blood / Milk and Food samples (raw and/or processed)

**Storage and Shelf life**

The provided kit has a shelf-life of 12 months when stored between -15°C to -25°C. Repeated thawing and freezing of PCR reagents should be avoided, as this may reduce the sensitivity. If the reagents are to be used multiple times, we recommend storing reagents as aliquots to avoid repeated freeze and thaw. Degradation of sample DNA specimens can also reduce the sensitivity of the assay. HiMedia Laboratories does not recommend using the kit after the expiry date stated on pack.

**Kit Contents:** The provided PCR kit contains:

| Components | Product code | Reagents provided for (reactions)* (µL) | |
|---|---|---|---|
| | | 25R | 50R |
| Hi-Quanti 4X Realtime PCR Mastermix | MBT200 | 169 µL | 338 µL |
| A1-A2 Primer-Probe Mix | DS1516 | 27 µL | 54 µL |
| Internal Control Primer-Probe Mix | DS1517 | 27 µL | 54 µL |
| Internal Control DNA | DS1096B | 27 µL | 54 µL |
| A1-A2 Positive Control | DS0781 | 25 µL | 50 µL |
| Molecular Biology Grade Water for PCR | ML065 | 325 µL | 650 µL |

***** For a 25 µL PCR reaction

**Specimen collection and Handling**

Follow appropriate techniques for handling specimens; after use, contaminated materials must be sterilized by autoclaving before discarding. Standard precautions as per established guidelines should be followed while handling clinical specimens and items contaminated with blood and other body fluids. Safety guidelines may be referred in individual safety data sheets.

**Sample Preparation**

Various milk samples are routinely examined. For extraction and purification of pure Milk DNA for high yield, perform the nucleic acid purification using HiMedia's extraction kits as instructed in the protocol.

**Materials needed but not provided**

- PCR tubes (Product code PW1255) or PCR Strips (Product code: PR17) or PCR Plates (Product code: PR2 / PR3 / PR19) & Sealing film (PR18)
- Insta Q Real Time PCR System (Product Code: LA1012 / LA1023 / LA1024 / LA1073 / LA1074)
- Barrier Micropipette Tips (Product Code: LA749 / LA749A / LA751 / LA751A / LA750 / LA750A / LA859 / LA859A)
- Micropipettes
- For Blood samples: HiPurA® Blood Genomic DNA Miniprep Purification Kit (MB504)
- For Meat / Tissue samples: HiPurA® Mammalian Genomic DNA Purification Kit (MB506)
- For Milk and Food samples (raw and/or processed): HiPurA® Food DNA Purification Kit (MB562)

**General Preparation Instructions**

- Before use all PCR components should be completely thawed on ice (4°C).
- Perform the amplification reactions in a clean area, preferably in a biosafety cabinet.
- Use of aerosol barrier pipette tips is recommended to reduce contamination risks from extraneous DNA templates.
- Extract and store positive control sample (if used) separately from all other reagents to avoid contamination and add it to the reaction mix in a separate area.

**A. Protocol for PCR Master Mix Preparation**

| Components | Product code | Volume (µL) to be added for 1R (for a 25 µL reaction) |
|---|---|---|
| Hi-Quanti 4X Realtime PCR Mastermix | MBT200 | 6.25 µL |
| A1-A2 Primer-Probe Mix | DS1516 | 1 µL |
| Internal Control Primer-Probe Mix | DS1517 | 1 µL |
| Internal Control DNA | DS1096B | 1 µL |
| Molecular Biology Grade Water for PCR | ML065 | 10.75 µL |
| Template DNA / Positive Control / Negative Control | - | 5 µL |
| Total volume | - | 25 µL |

**Note: The template volume can be increased upto 10 µL by adjusting the volume of Molecular Biology Grade Water for PCR.**

Centrifuge the tube briefly at 6000 rpm for about 10 seconds. Place the tubes in Real-time PCR machine and set the recommended PCR program (mentioned below). Interpret the data from the amplification plot (observe the Ct values).

**B. Recommended PCR program**

| | | | | |
|---|---|---|---|---|
| 1. | UNG Incubation | : | 25°C for 2 minutes | |
| 2. | Initial denaturation | : | 95°C for 2 minutes | |
| 3. | Denaturation | : | 95°C for 10 seconds | No. of cycles: 40 |
| 4. | Annealing & Extension | : | 50°C for 30 seconds (Sampling) | |
| | Channels | : | FAM/JOE/TexasRed | |
| 5. | Hold | : | 4°C for ∞ | |

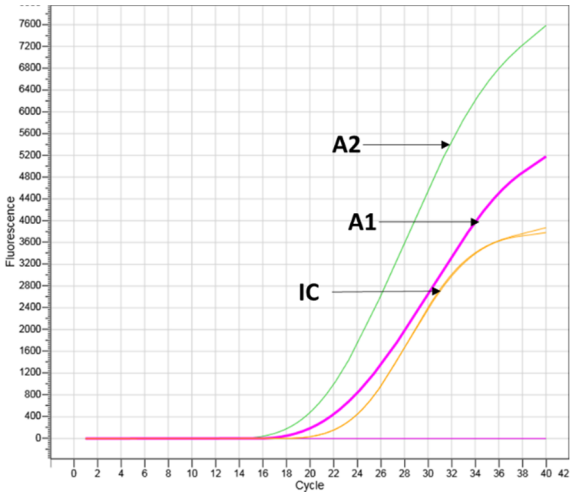
## C. Data Analysis

The following conditions should be met for a valid diagnostic test:

| Control | Detection channel | | |
|---|---|---|---|
| | FAM (A2) | JOE (A1) | Texas Red (Internal Control) |
| Positive Control | + | + | + |
| Negative Control | - | - | + |

| Ct value | Result |
|---|---|
| ≤ 36 | Detected (+) |
| > 36 or N/A | Not detected (-) |

## D. Amplification Data




| Target | Positive Control | Negative Control |
|---|---|---|
| A1 | 20.28 | - |
| A2 | 20.12 | - |
| IC | 23.87 | 23.88 |

**Image representing amplification plot of A1 and A2 DNA with Ct values using HiMedia's Hi-PCR® A1A2 Probe PCR Kit. The results completely depend upon sample types.**

## E. Data Interpretation

| Detection Channel | | | Result Interpretation |
|---|---|---|---|
| FAM (A2) | JOE (A1) | Texas Red (Internal Control) | |
| + | - | +/-* | Positive for A2A2 genotype |
| - | + | +/-* | Positive for A1A1 genotype |
| + | + | +/-* | Positive for A1A2 genotype |
| - | - | +/-* | Negative for A1 or A2 genotype |
| - | - | - | PCR inhibition or reagent failure. Repeat PCR or repeat extraction from original sample |

*The presence or absence of a signal in the Texas Red channel is not relevant for the validity of the test run due to competition between the test template and Internal Control template.

**Analytical Performance**

**Limit of Detection (LoD) - Analytical Sensitivity**

Sensitivity for the HiMedia's Hi-PCR® A1A2 Probe PCR Kit was conducted on InstaQ96® Real Time PCR system. The detectable limit of the HiMedia's Hi-PCR® A1A2 Probe PCR Kit was determined to be **3 copies/µl**. The analytical sensitivity of HiMedia's Hi-PCR® A1A2 Probe PCR Kit for mixture of A1 in A2 was also evaluated. This was determined by combining A1 and A2 DNA in pre-determined proportion, from 100% A1 to 100% A2 (as shown below). The limit of detection **of A1 in A2** for HiMedia's Hi-PCR® A1A2 Probe PCR Kit was determined to be **7% (41 copies)**.

| Percentage of A1 in A2 | | Difference in Ct ± SD |
|---|---|---|
| **A1** | **A2** | |
| 100 | 0 | -- |
| 90 | 10 | -4.38 ± 0.12 |
| 80 | 20 | -2.15 ± 0.06 |
| 70 | 30 | -0.65 ± 0.11 |
| 60 | 40 | 0.25 ± 0.11 |
| 50 | 50 | 1.36 ± 0.03 |
| 40 | 60 | 2.12 ± 0.08 |
| 30 | 70 | 3.63 ± 0.18 |
| 20 | 80 | 5.05 ± 0.45 |
| 10 | 90 | 7.61 ± 0.97 |
| 8 | 92 | 8.77 ± 0.98 |
| **7** | **93** | **9.32 ± 0.83** |
| 5 | 95 | 10.86 ± 0.86 |
| 2 | 98 | Not Detected |
| 1 | 99 | Not Detected |
| 0.5 | 99.5 | Not Detected |
| 0 | 100 | -- |

**Inclusivity**

*In silico* analysis for the assessment of inclusivity for the HiMedia's Hi-PCR® A1A2 Probe PCR Kit was conducted by mapping the primers and probe against the available A2 and A1 sequences in GenBank. The HiMedia's Hi-PCR® A1A2 Probe PCR Kit targets 100% of the known A1 and A2 positive strains.

**Cross-reactivity - Analytical Specificity**

*In silico* analysis was performed using NCBI nucleotide and Primer BLAST. The primers and probe for A1 and A2 region were analyzed against organisms that are most frequently encountered.

**Warning**

Not for Medicinal Use.

**Precautions**

Read the procedure carefully before starting the experiment. Wear protective gloves/protective clothing/eye protection/face protection. Follow good clinical laboratory practices while handling clinical samples. Standard precautions should be followed as per established guidelines. Safety guidelines may be referred to in safety data sheets of the product.

**Limitations**

Although rare, mutations within the highly conserved regions of the targets genes covered by the kit's primers and/or probe may result in under quantitation or failure to detect the presence of the target regions in these cases. Validity and performance of the assay design are revised at regular intervals.

**Performance and Evaluation**

Each lot of HiMedia's Hi-PCR® A1A2 Probe PCR Kit is tested against predetermined specifications to ensure consistent product quality.

**Quality Control**

Each lot of HiMedia's Hi-PCR® A1A2 Probe PCR Kit is assayed for contaminating endonuclease, exonuclease and non-specific DNase activities. Functionally tested in DNA amplification.

**Troubleshooting Guide**

| Sr. No. | Problem | Cause | Solution |
|---|---|---|---|
| 1. | No amplification | Degraded samples | 1. Check the integrity of DNA using agarose gel electrophoresis. |
| | | | 2. Use freshly prepared DNA to ensure the availability of intact template sequence for efficient amplification. |
| | | Error in protocol setup | Verify that the correct reagent volumes, dilutions and storage conditions have been used. |
| 2. | Variability between replicates | Error in reaction set-up | Prepare a large volume master mix, vortex thoroughly and aliquot into reaction tubes. |
| | | Air bubbles in reaction mix | Briefly centrifuge reaction samples/plate prior to running on a real-time PCR instrument. |
| | | Pipetting error | $C_t$ values of replicates can show increased variation due to poor laboratory technique or imprecise pipettes. |
| 3. | Amplification in negative control | Reagents contaminated | 1. Replace all critical solutions.<br>2. Repeat the analysis of all tests with fresh aliquots of critical reagents. |
| 4. | No signal with positive controls | Incorrect programming of the temperature profile of the thermocycler | Compare the temperature profile to the manual. |

**Safety Information**

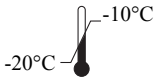
HiMedia's Hi-PCR® A1A2 Probe PCR Kit is for laboratory use only, not for drug, household or other uses. Take appropriate laboratory safety measures and wear gloves when handling.

**Disposal**

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques.

**Technical Assistance**

At HiMedia, we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com.




Storage temperature

Do not use if package is damaged

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