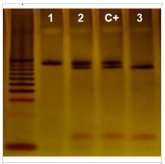

Apr 12, 2021

Genotyping of polymorphisms rs1801133 in *MTHFR*, rs1051266 in *SLC19A1* and rs1805087 in *MTR*

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LPM UFG

Nayane Soares de Lima

ABSTRACT

The metabolic cycle of folate, also known as one-carbon metabolism, is a set of interconnected pathways that provide methyl groups for essential metabolic functions in the body. This cycle is responsible for crucial reactions in intracellular homeostasis such as methylation of biomolecules as well as synthesis of nucleic acids and amino acids. It is also important in regulating the levels of folate, homocysteine (Hcy) and methionine within cells. The *MTHFR*, *SLC19A1, MTR* genes are responsible for intracellular regulation and plasma transport of folate and for remethylation of Hcy in methionine, respectively. The presence of polymorphisms in these genes can cause a reduction in serum folate levels as well as raise plasma levels of Hcy, inducing a neurotoxic picture, Hyperhomocysteinaemia, which is a contributing factor to the development of neurodegenerative diseases. Thus, this protocol provides a standardization of the genotyping of polymorphisms rs1801133 in *MTHFR*, rs1051266 in *SLC19A1* and rs1805087 in *MTR* through the polymerase chain reaction (PCR) followed by the restriction fragment length polymorphism technique (PCR-RFLP). The PCR-RFLP technique is widely used for genotyping as it is a simple, low cost and high reproducibility procedure facilitating the analysis of genetic association of several polymorphisms with a variety of diseases.

DOI

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MATERIALS TEXT

Reagents for PCR master mixing:

10X PCR Buffer Sinapse INC ®
MgCl2 50mM Sinapse INC ®
dNTPs Sinapse INC ®
Taq Polymerase Sinapse INC ®
Primers (mix sense and antisense) Integrated DNA Technologies ® (IDT)

Reagents for RFLP mixing:

Enzyme buffer (Thermo Fisher Scientific ™)
Gene *SLC19A1/RFC1*: *HhaI* Restricted Enzyme (Thermo Fisher Scientific ™)
Gene *MTHFR: HinfI* Restricted enzyme (Thermo Scientific ™)
Gene *MTR*: *HaeIII* Restricted Enzyme (SibEnzyme®)
Enzyme buffer (SibEnzyme®)

Reagents for electrophoresis run:

Loading dye (Sinapse INC ®)
100bp DNA ladder (Sinapse INC ®)
50bp DNA ladder (Sinapse INC ®)
20bp DNA ladder (BIO-RAD ®)

Reagents for Polyacrylamide gel:

Reagents	1 gel	2 gels
Acrylamide	4.5ml	9ml
Tris-Borate-EDTA (TBE) 10x	8ml	16ml
Distilled H20	2.25µL	4.5µL
Ammonium persulfate (APS) 10%	225µL	450µL
Tetramethylethylenediamine (TEMED)	22.5µL	45µL

The concentration of the gel may vary according to the molecular weight of its fragments of interest.

Fast Silver Nitrate staining method:

00:05:00 After adding the solution to the gel, mix the reaction for at least a few minutes.

Reagents	Vol.
Distilled water	248ml
Acetic acid	2ml
Ethl alcohol	50ml
Final volume	300ml

Prepare fixing solution 25% (preparation at time of use)

Revelation solution:

Reagents	Vol.
NaOH 30%	4.5ml
Formaldehyde 37%	3ml
Distilled water	200ml

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Apply to the gel after silver nitrate colouring and then washing the gel

SAFETY WARNINGS

Always be careful when handling any kind of reagent. For most of these chemical substances are harmful to humans. And always before starting any experiment, make sure to follow the biosafety protocols of your laboratory.

DISCLAIMER:

BEFORE STARTING

The use of personal protective equipment is required and indispensable in the laboratory environment.

DNA extraction

1

For handling any biological material, remember to always follow your laboratory's biosafety standards.

The DNA samples were extracted from peripheral blood and submitted to DNA purification using the commercial extraction kit, following the protocol suggested by the manufacturer (Purilink Invitrogen® USA). The extracted DNA samples were labeled and stored in a -20°C freezer.

1.1 After purification of the genomic material, the samples were subjected to quality evaluation by spectrophotometry NanoDrop™ ND-1000 (ThermoFisher®, USA). They were then subjected to PCR molecular techniques followed by RFLP for analysis of the polymorphisms in question.

Gene *MTHFR*

2 O *Methylenetetrahydrofolate reductase* (*MTHFR*) is the gene responsible for encoding an enzyme of the same name, fundamental in the one-carbon metabolism. This enzyme regulates the intracellular biochemical pathways of the folate by converting 5.10-MTHF to 5-MTHF, acting on essential processes such as the synthesis of nucleotides,

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

methionine and other proteins fundamental to the body. MTHFR directly involves the regulation of this metabolic pathway, due to the synthesis of the main substrate for the transfer of the methyl grouping in the process of remetillation of Hcy into methionine (Botto and Yang 2000).

Botto, L. D., & Yang, Q. (2000). 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *American journal of epidemiology*, *151*(9), 862–877. https://doi.org/10.1093/oxfordjournals.aje.a010290.

2.1 Design of primers

The primers previously described by Keku *et al.* (2002) were used to amplify the region of interest of the *MTHFR* gene. The sequences of the primers were:

Forward 5'-AGGCTGACCTGAAGCACTTGAA-3'
Reverse 3'-CTCAAAGAAAAGCTGCGTGATGA-5'

Keku, T., Millikan, R., Worley, K., Winkel, S., Eaton, A., Biscocho, L., Martin, C., & Sandler, R. (2002). 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, *11*(12), 1611–1621.

2.2

Mandatory use of jacket, gloves and mask for this procedure. They are necessary to avoid contamination of the running reaction.

PCR amplification

A conventional polymerase chain reaction (PCR) was performed to amplify the 198 base pair (bp) region of the *MTHFR* . The PCR reagent mix had a total volume of 25 µL per reaction. All PCR reagents were from Sinapse INC®.

Of which:

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

Reagents	Final concentration	Final volume
10X PCR buffer solution	1x	2.5 µL
dNTP mixture 10 mM	0.2mM	0.5 µL
Primer mixture, containing primer sense (2.5 mM) and antisense primer (2.5 mM)	0.1mM	1.0 µL
MgCl2 50 mM	10mM	0.5 µL
Taq DNA polymerase (5U/µL)	1.25U/µL	0.5 µL
Milli-Q water	-	18.0 µL
DNA	-	2.0 µL

All reactions to a total volume of 25 µL.

The PCR amplification was performed with the Bio-rad thermal cycler (T100™ Thermal Cycler). Cycling conditions were 94°C for 5 min for initial denaturation, followed by 36 cycles of denaturation at 94°C for 1 min, hybridization of primers at 61°C for 1 min and extension at 72°C for 1:30 min. A final stage of 72°C for 7 minutes was performed to complete the elongation processes and maintained at 4°C at the end.

For the PCR fragments visualization, a mix of 6 µl amplicons and 4 µl 6× DNA running buffer (loading Dye) applied in 15% polyacrylamide gel, running in TBE 1X buffer was performed. A molecular-based marker (100bp) was used as a reference. After 240 min under 80 Volts and 60 Milliamperes, the polyacrylamide gel was stained with silver nitrate. The presence of a 198bp fragment confirmed the amplification of the PCR product, allowing subsequent steps to be performed. Negative controls (DNA-free PCR mix) were included in all PCR and electrophoresis assays.

2.3

Mandatory use of gloves and mask for this procedure. They are necessary to avoid contamination of the running reaction.

Genotyping of polymorphism rs1801133 in *MTHFR* by PCR-RFLP

The 198pb amplicons were digested with the restriction enzyme *HinfI* (Thermo Scientific™). It has a concentration of 10U/uL. It is derived from the organism *Haemophilus influenza* and should be kept at -20°C.

The restriction was performed using the Bio-rad thermal cycler (T100™ Thermal Cycler) under the following cycling conditions: 6 h (37°C), 20 min (80°C) and hold at 4°C.

They contained:

Reagents	Volume
Buffer R 10X	2 µL
Milli-Q Water	17.5 µL
Restriction enzyme	0.5 µL
Amplified product	10 µL

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

All reactions to a total volume of 30 µL.

After the restriction stage, 10 µL of the digested product and 4 µL of 6× loading buffer was applied in 15% polyacrylamide gel with running in TBE 1X buffer solution. A molecular weight marker (20bp) was used as reference. After 360 min under 80 Volts and 60 Miliamperes, the polyacrylamide gel was stained with silver nitrate. In all electrophoresis assays, positive controls (sample previously known as heterozygous genotype) were included.

2.4

Beware when handling all kinds of reagents, because when handling incorrectly, accidents can occur!

Analysis and interpretation of results

Following the above steps, the interpretation of the genotyping of polymorphism rs1801133 in *MTHFR* is based on the activity of the enzyme *HinfI*. It recognizes the restriction site (5'-G | ANT C-3') (3'-C TNA | G-5') and promotes a cut in the fragment of 198bp, providng the digestion of the same, generating three fragments (198, 175, and 23bp).

Thus, the wild genotype (CC) presents the fragments of 198bp. The heterozygous genotype (CT) is determined by the presence of three fragments (198, 175, and 23bp). And for the mutant genotype (TT) two fragments (175, and 23bp). Presented in Figure 1.

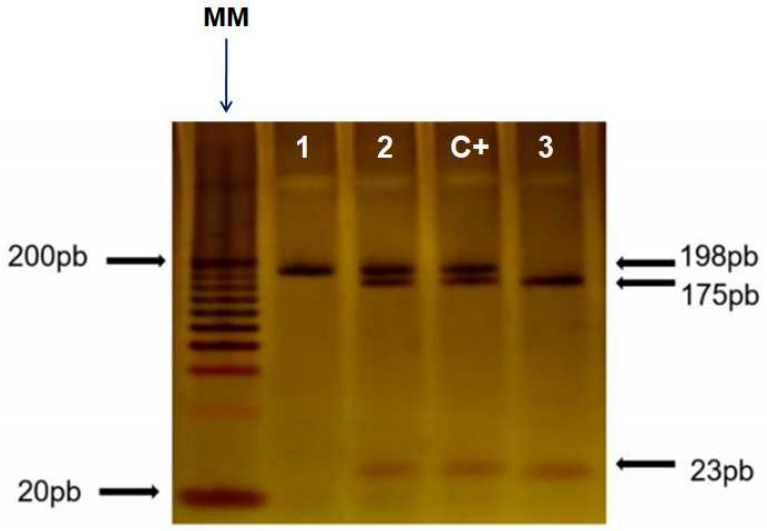

Figure 1. Original gel from the genotyping of polymorphism rs1801133 of *MTHFR* by PCR-RFLP. **MM**: molecular weight marker (20bp - BioRad®). **1**: Wild (C/C). **2:** Heterozygoto (C/T); **C+:** positive control; **3**: Mutant (T/T);

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

Gene *SLC19A1/RFC1*

3 The *SLC19A1* gene, the reduced folate carrier, also known as *RFC1*, is responsible for encoding a membrane protein responsible for ubiquitous folate transport, playing an important role in maintaining its intracellular concentration.When at low expression levels due to some kind of alteration, or as a non-functional carrier can be related to cardiovascular diseases, fetal aberrations, neurological alterations and even cancer (Hou and Matherly 2014).

Hou, Z., & Matherly, L. H. (2014). Biology of the major facilitative folate transporters SLC19A1 and SLC46A1. *Current topics in membranes*, *73*, 175–204. https://doi.org/10.1016/B978-0-12-800223-0.00004-9.

3.1 Design of primers

The primers previously described by Zara-Lopes *et al.* (2019) were used to amplify the region of interest of the *SLC19A1/RFC1* gene. The sequences of the primers were:

Forward 5' - AGT GTC ACC TTC GTC CC - 3'
Reverse 3' - TCC CGC GTG AAG TTC TTG - 5'

Zara-Lopes, T., Galbiatti-Dias, A., Castanhole-Nunes, M., Padovani-Júnior, J. A., Maniglia, J. V., Pavarino, E. C., & Goloni-Bertollo, E. M. (2019). Polymorphisms in *MTHFR*, *MTR*, *RFC1* and *CßS* genes involved in folate metabolism and thyroid cancer: a case-control study. *Archives of medical science : AMS*, *15* (2), 522–530. https://doi.org/10.5114/aoms.2018.73091.

3.2 PCR amplification

A conventional polymerase chain reaction (PCR) was performed to amplify the 230 base pair (bp) region of the *SLC19A1/RFC1* gene. The PCR reagent mix had a total volume of 25 µL per reaction. All PCR reagents were Sinapse INC®.

Of which:

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

Reagents	Final concentration	Volume
10X PCR buffer solution	1x	2.5 µL
dNTP mixture 2.5 mM	0.2mM	1.5 µL
Primer mixture, containing primer sense (2.5 mM) and antisense primer (2.5 mM)	0.1mM	1.5 µL
MgCl2 50 mM	1.0mM	0.7 µL
Taq DNA polymerase (5.0 U/µL)	1.25U/µL	0.5 µL
Milli-Q water	-	14.3 µL
DNA	-	4.0 µL

All reactions to a total volume of 25 µL.

The PCR amplification was performed with the Bio-rad thermal cycler (T100^{TM} Thermal Cycler). Cycling conditions were 94°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, hybridization of primers at 60°C for 1 min and extension at 72°C for 1:30 min. A final stage of 72°C for 7 minutes was performed to complete the elongation processes and maintained at 4°C at the end.

For the PCR fragments visualization, a mix of 6 µl amplicons and 4 µl 6× DNA running buffer (loading Dye) applied in 12% polyacrylamide gel, running in TBE 1X buffer was performed. A molecular-based marker (100bp) was used as a reference. After 240 min under 80 Volts and 60 Milliamperes, the polyacrylamide gel was stained with silver nitrate. The presence of a 230bp fragment confirmed the amplification of the PCR product, allowing subsequent steps to be performed. Negative controls (DNA-free PCR mix) were included in all PCR and electrophoresis assays.

3.3 Genotyping of polymorphism rs1051266 in *SLC19A1* by PCR-RFLP

The 230pb amplicons were digested with the restriction enzyme *HhaI* (Thermo Scientific^{TM}). It has a concentration of 10U/uL. It is derived from the organism *Haemophilus haemolyticus* and should be kept at -20°C.

The restriction was performed using the Bio-rad thermal cycler (T100^{TM} Thermal Cycler) under the following cycling conditions: 6 h (37°C), 20 min (80°C) and hold at 4°C.

They contained:

Reagents	Volume
SE-Buffer G 10X	2 µL
Milli-Q Water	17.5 µL
Restriction enzyme	0.5 µL
Amplified product	10 µL

All reactions to a total volume of 30 µL.

After the restriction stage, 10 µL of the digested product and 4 µL of 6× loading buffer was applied in 15% polyacrylamide gel with running in TBE 1X buffer solution.

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

A molecular weight marker (20bp) was used as reference. After 360 min under 80 Volts and 60 Miliamperes, the polyacrylamide gel was stained with silver nitrate. In all electrophoresis assays, positive controls (sample previously known as heterozygous genotype) were included.

3.4 Analysis and interpretation of results

Following the above steps, the interpretation of the genotyping of polymorphism rs1051266 in *SLC19A1* is based on the activity of the enzyme *HhaI*. It recognizes the restriction site (5'...GC G↓C...3')(3'... C↑G CG...5') and promotes a cut in the fragment of 230bp, providng the digestion of the same, generating four fragments (162, 125, 68 and 37bp).

Thus, the wild genotype (AA) presents the fragments of 162 and 68bp. The heterozygous genotype (GA) is determined by the presence of four fragments (162, 125, 68 and 37bp). And for the mutant genotype (GG) three fragments (125, 68 and 37bp). Presented in Figure 2.

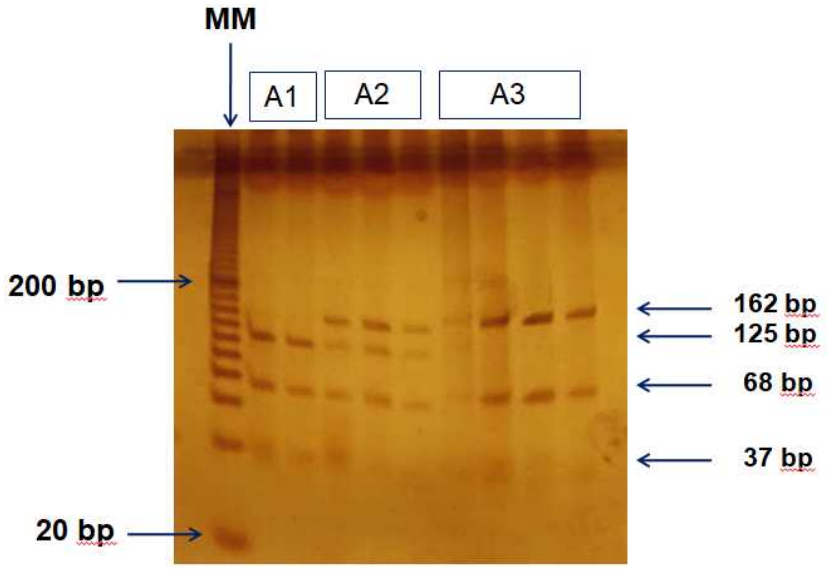

Figure 2. Original gel from the genotyping of polymorphism rs1051266 of *SLC19A1* by PCR-RFLP. **MM**: molecular weight marker (20bp - BioRad®). **A1**: Mutant G/G; **A2**: Heterozygoto A/G and positive control; **A3**: Wild A/A.

Gene *MTR*

4 The gene 5-methylenotetrahydrofolate-homocysteine methyltransferase is also known as methionine synthase (*MTR*). This gene is responsible for encoding a homonymous protein which acts as a cytoplasmic enzyme, with active action one-carbon metabolism. It is responsible for catalyzing the biosynthesis of homocysteine into methionine in the methylation pathway from the donation of a 5-tetrahydrofolate methyl group to Hcy. It is also important to maintain adequate levels of intracellular methionine, a precursor amino acid of S-adenosylmethionine

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

(SAM), which in turn is crucial in methylation reactions of biomolecules such as DNA, RNA, lipids and proteins (Ma *et al.* 1999).

Ma, J., Stampfer, M. J., Christensen, B., Giovannucci, E., Hunter, D. J., Chen, J., Willett, W. C., Selhub, J., Hennekens, C. H., Gravel, R., & Rozen, R. (1999). A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocyst(e)ine, and colorectal cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*,*8*(9), 825–829.

4.1 Design of primers

The primers previously described by Zara-Lopes *et al.* (2016) were used to amplify the region of interest of the *MTR* gene. The sequences of the primers were:

Forward 5' - CCA GGG TGC CAG GTA TAC AG -3'
Reverse 3' - GCC TTT TAC ACT CCT CAA AAC -5'

Zara-Lopes, T., Gimenez-Martins, A. P., Nascimento-Filho, C. H., Castanhole-Nunes, M. M., Galbiatti-Dias, A. L., Padovani-Júnior, J. A., Maniglia, J. V., Francisco, J. L., Pavarino, E. C., & Goloni-Bertollo, E. M. (2016). Role of MTHFR C677T and MTR A2756G polymorphisms in thyroid and breast cancer development. *Genetics and molecular research : GMR*,*15*(2), 10.4238/gmr.15028222. https://doi.org/10.4238/gmr.15028222.

4.2 PCR amplification

A conventional polymerase chain reaction (PCR) was performed to amplify the 498 base pair (bp) region of the *MTR* gene. The PCR reagent mix had a total volume of 25 µL per reaction. All PCR reagents were Sinapse INC®.

Of which:

Reagents	Final concentration	Volume
10X PCR buffer solution	1x	2.5 µL
dNTP mixture 2.5 mM	0.2mM	1.0 µL
Primer mixture, containing primer sense (2.5 mM) and antisense primer (2.5 mM)	0.1mM	1.0 µL
MgCl2 50 mM	1.0mM	0.5 µL
Taq DNA polymerase (5.0 U/µL)	1.25U/µL	0.5 µL
Milli-Q water	-	15.5 µL
DNA	-	4.0 µL

All reactions to a total volume of 25 µL.

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The PCR amplification was performed with the Bio-rad thermal cycler (T100^{TM} Thermal Cycler). Cycling conditions were 94°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, hybridization of primers at 60°C for 1 min and extension at 72°C for 1:30 min. A final stage of 72°C for 7 minutes was performed to complete the elongation processes and maintained at 4°C at the end.

For the PCR fragments visualization, a mix of 6 µl amplicons and 4 µl 6× DNA running buffer (loading Dye) applied in 12% polyacrylamide gel, running in TBE 1X buffer was performed. A molecular-based marker (100bp) was used as a reference. After 240 min under 80 Volts and 60 Milliamperes, the polyacrylamide gel was stained with silver nitrate. The presence of a 498bp fragment confirmed the amplification of the PCR product, allowing subsequent steps to be performed. Negative controls (DNA-free PCR mix) were included in all PCR and electrophoresis assays.

4.3 Genotyping of polymorphism rs1805087 in *MTR* by PCR-RFLP

The 498pb amplicons were digested with the restriction enzyme *HaeIII* (SibEnzyme®). The restriction enzyme contains 2,000 units at 10,000u/ml. It is derived from the organism *Haemophilus aegyptius* and should be kept at -20°C.

The restriction was performed using the Bio-rad thermal cycler (T100^{TM} Thermal Cycler) under the following cycling conditions: 6 h (37°C), 20 min (80°C) and hold at 4°C.

They contained:

Reagents	Volume
SE-Buffer G 10X	2 µL
Milli-Q Water	17.5 µL
Restriction enzyme	0.5 µL
Amplified product	10 µL

All reactions to a total volume of 30 µL.

After the restriction stage, 10 µL of the digested product and 4 µL of 6× loading buffer was applied in 15% polyacrylamide gel with running in TBE 1X buffer solution.

A molecular weight marker (20bp e 50bp) was used as reference. After 360 min under 80 Volts and 60 Miliamperes, the polyacrylamide gel was stained with silver nitrate. In all electrophoresis assays, positive controls (sample previously known as heterozygous genotype) were included.

4.4 Analysis and interpretation of results

Following the above steps, the interpretation of the genotyping of polymorphism rs1805087 in *MTR* is based on the activity of the enzyme *HaeIII*. It recognizes the restriction site (5'...GG↓CC...3')(3'...CC↑GG...5') and promotes a cut in the fragment of 498bp, providng the digestion of the same,

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w

generating four fragments (413, 290, 123 and 85bp).

Thus, the wild genotype (AA) presents the fragments of 413 and 85bp. The heterozygous genotype (GA) is determined by the presence of four fragments (413, 290, 123 and 85bp). And for the mutant genotype (GG) three fragments (290, 123 and 85bp). Presented in Figure 3.

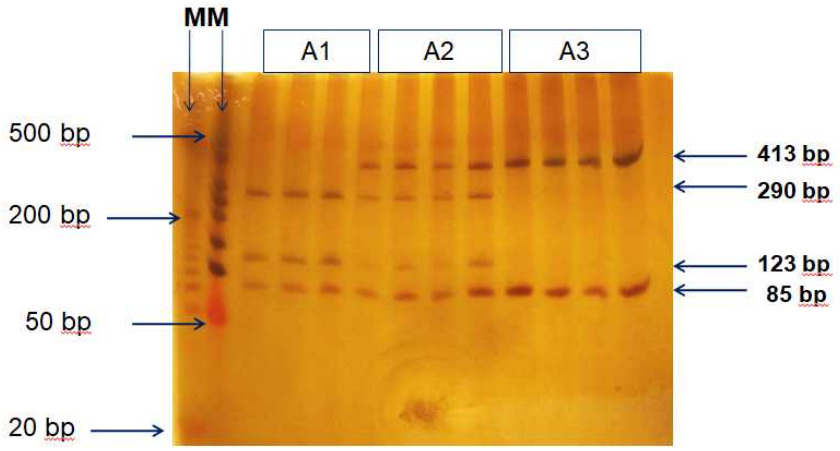

Figure 3. Original gel from the genotyping of polymorphism rs1805087 of *MTR* by PCR-RFLP. **MM**: molecular weight marker (20bp - BioRad® and 50bp Sinapse®). **A1**: Mutant G/G; **A2**: Heterozygoto A/G and positive control; **A3**: Wild A/A.

Citation: Nayane Soares de Lima, Caroline Christine Pincela da Costa , RÃÂ´mulo Morais Azevedo, Angela Adamski S Reis, Rodrigo da Silva Santos (04/12/2021). Genotyping of polymorphisms rs1801133 in MTHFR, rs1051266 in SLC19A1 and rs1805087 in MTR . https://dx.doi.org/10.17504/protocols.io.brrkm54w