

Association of Glycoprotein GP130 Polymorphisms (rs747379809, rs754547662) with Unexplained Infertility in Women Using TETRA-ARMS-PCR

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ABSTRACT

Objective: To analyse the association of single-nucleotide polymorphisms in two variants of the GP130 (*IL6ST*) gene, rs754547662 Guanine/thymine (G/T) and rs747379809 Guanine/thymine (G/T), with unexplained infertility in women.

Study Design: A case-control study.

Place and Duration of the Study: Department of Physiology, University of Karachi, in collaboration with the Australian Concept of Infertility Medical Centre, from November 2020 to December 2022.

Methodology: A total of 135 females with unexplained infertility and 177 fertile women serving as controls were enrolled in the study. Genotyping was performed using Tetra-Amplification Refractory Mutation System Polymerase Chain Reaction (T-ARMS-PCR). Genomic DNA was amplified by T-ARMS-PCR, followed by agarose gel electrophoresis for the identification of the tested allele. The amplified and purified samples were sequenced *via* Sanger sequencing and subsequently submitted to GenBank. All statistical analyses were performed using SPSS 23. An independent t-test was used for group comparison; $p < 0.05$ was considered statistically significant.

Results: In the rs747379809 and rs754547662 variants of the GP130 (*IL6ST*) gene, only the wild-type G allele was detected in all cases and controls. The major allele G and the GG (homozygous) genotype were found in all women. The minor allele T and the GT, TT (heterozygous) genotypes were not detected in any individual. Therefore, the findings reported no observed association between GP130 (*IL6ST*) gene polymorphisms rs747379809 (G/T) and rs754547662 (G/T) and unexplained infertility.

Conclusion: The findings indicate that unexplained infertility is not associated with the GP130 (*IL6ST*) gene polymorphisms in rs747379809 and rs754547662 variants. These results suggest that variations in this signalling pathway do not contribute significantly to the aetiology of unexplained infertility in the studied population. Further research involving larger cohorts and additional molecular markers may help clarify the complex genetic landscape underlying this condition.

Key Words: Embryo Implantation, Genetic Polymorphism, Glycoprotein, GP130 (*IL6ST*), Infertility.

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INTRODUCTION

Couples failing to conceive without any specific cause are considered to have unexplained infertility (UE-IF).¹ UE-IF reflects a woman's inability to conceive after twelve cycles of contraceptive-free coitus despite normal assessments.² Relying solely on normal findings and standard investigations poses a challenge for infertility experts, as many parameters necessary to assess fecundity have not been thoroughly explored in the context of UE-IF.³ Consequently, UE-IF is a major global issue with serious emotional repercussions for affected couples.³

Its worldwide prevalence ranges from 8–37%. In the Middle East and nearby/peripheral countries, infertility affects around 38.5% of couples, with 22.6% of cases unexplained.⁴ This shows the need for better management of affected couples.⁴

In women with UE-IF, suboptimal endometrial receptivity is increasingly recognised as a major reason hindering embryo implantation, even when hormonal response, embryo quality, endometrial development, and routine clinical evaluations appear normal.^{5,6} Assisted Reproductive Technologies (ART) continue to be used as the final treatment option for UE-IF despite rising rates of unexplained implantation failure.^{5,6} The implantation process requires the interaction of cytokine leukaemia inhibitory factor (LIF) and its receptors, LIFR and glycoprotein GP130 (*IL6ST*), a signal transducer component of LIFR.⁶ A clear understanding of the implantation pathway governed by LIFR and its co-receptor GP130 (*IL6ST*), which plays a central role in establishing endometrial receptivity to the blastocyst, is crucial for improving outcomes in UE-IF.⁷

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Glycoprotein GP130 (*IL6ST*) is located at the tip of the endometrium on specialised structures called pinopods, where it functions as a critical component of LIFR.⁸ The expression and activation of GP130 (*IL6ST*) by progesterone and LIF facilitate signal transduction, which activates the JAK-STAT signalling pathway in the uterus.⁸ This mechanism guides the embryo towards the LIF receptor complex LIFR-GP130 on pinopods, enabling successful implantation.⁹ During the implantation window, GP130 (*IL6ST*) and LIFR are predominantly expressed on the blastocyst's surface and the endometrial epithelium.⁹ Approximately two-thirds of implantation failures are attributed to dysfunction of the GP130 (*IL6ST*) component, resulting in aberrant signalling between the embryo and the endometrial lining.⁸

Although scientists discovered the role of the LIFR gene polymorphism in infertility,¹⁰ far less attention has been paid to its co-receptor, the GP130 (*IL6ST*) gene polymorphism, particularly in relation to infertility.¹¹ The current study focused on the GP130 (*IL6ST*) gene polymorphism because the implantation process, activated through LIFR and mediated by GP130 (*IL6ST*), remains insufficiently addressed, and its genetic polymorphisms remain largely uninvestigated in women with UE-IF. To address this gap, the present study aimed to investigate whether the GP130 (*IL6ST*) gene polymorphism contributes to UE-IF. The objective was to analyse the association of Single Nucleotide Polymorphisms (SNP) in the two GP130 (*IL6ST*) gene variants, rs754547662 Guanine/thymine (G/T) and rs747379809 Guanine/thymine (G/T). Both variants have a severe consequence type: stop codon. This study thus aimed to provide early insights into a potential genetic marker and would serve as a critical step towards addressing a broader clinical challenge in infertility, enhancing diagnostic precision and guiding future research in implantation biology.

METHODOLOGY

A case-control study was carried out at the Department of Physiology, University of Karachi, in collaboration with the Australian Concept of Infertility Medical Centre and Urban Hospital, Pakistan, from November 2020 to December 2022. Ethical approval was obtained from the Institutional Bioethics Review Committee of the University of Karachi, Karachi, Pakistan (Approval No. IBC124-B/2020). Samples for the study were obtained from the Australian Concept of Infertility Medical Centre and Urban Hospital, Pakistan. All experimental procedures were conducted in accordance with the STROBE guidelines and the ethical principles outlined in the Declaration of Helsinki.

OpenEpi (www.openepi.com) was utilised to calculate the required sample size to achieve 80% statistical power, with a 5% margin of error, and to detect an odds ratio of at least 2 among women diagnosed with UE-IF.¹² A two-sided signifi-

cance level of 5% was applied; the estimated sample size was 81 cases and 162 controls ($n = 243$). The sample size was expanded to include 135 women with UE-IF and 177 fertility-proven controls ($n = 312$), enhancing the robustness and precision of the study. This deviation did not introduce selection bias because cases and controls continued to meet the predefined inclusion criteria and were recruited from the same source population.

Participants were recruited through a convenience sampling technique, including all eligible women who voluntarily presented and met the study criteria during the data collection period. A total of 177 women with proven fertility were used as a control group based on normal cycles, hormonal reports, and ultrasound findings. All study participants were age- and BMI-matched. Written informed consent was obtained from all research participants.

Based on the inclusion criteria, 135 women having UE-IF within the age group of 18-40 years and a history of >18-24 months of infertility, regular menstrual cycles (25-35 days), and hormonal signs of ovulation were recruited. Assessments to qualify women as cases included normal thyroid-stimulating hormone (TSH) levels, follicle-stimulating hormone (FSH) less than 11IU/L in the early follicular phase, and serum prolactin concentrations below 20mg/L. The body mass index (BMI) range was 18-25 kg/m². Their husbands had a normal seminal profile. Pelvic ultrasonography formed part of the inclusion and exclusion criteria; only women with normal ultrasound findings, indicating healthy reproductive organs, were included. Women with gynaecological, metabolic, or medical problems, or with structural abnormalities such as fibroids, ovarian cysts, hydrosalpinx, or uterine anomalies identified on pelvic ultrasound, or who were using contraceptives, were excluded.

Hormonal assays and routine laboratory parameters of participants were verified from the hospital's diagnostic records under the supervision of consultants, including serum TSH, FSH <11 IU/L in the early follicular phase, prolactin (<20 ng/mL), haemoglobin, haematocrit, HbA1c, urea, sodium, and potassium levels. Semen analysis for partners of infertile women was reviewed using reports available in the hospital records, as per WHO criteria.¹³ These parameters were assessed to exclude metabolic, endocrine, haematological, or renal abnormalities that could independently affect reproductive function. This ensured that all participants met the criteria for unexplained infertility and that fertile controls had normal baseline physiological profiles.

Body fat percentage was measured using a bioelectrical impedance analyser (Tanita Corp., Tokyo, Japan). Anthropometric indices, including BMI, and baseline clinical parameters such as blood pressure and pulse rate were recorded using a structured study proforma. The data collectors were blinded to the status of the case-control study.

Genomic DNA (gDNA) was extracted using an inexpensive, high-throughput salting-out technique, with all steps following the original protocol.¹⁴ gDNA was also isolated using the Wizard[®] Genomic DNA Purification Kit (Promega, Technical Manual Cat# A1125, US), which employs a protein-precipitation method to obtain high-quality gDNA, and was refrigerated at -86 °C for genotyping. Subsequently, gel electrophoresis was used to validate 1 µL of each of the DNA samples (90%) by applying 1% agarose gel for quality check. The absorbance ratio (A_{280}/A_{260}) was calculated for 2 µL samples using ND-1000 V3.8.1 soft-ware (Thermo Fisher Scientific, Waltham, MA). The ratio measured 1.8 was accepted to validate the purity of gDNA. Genotyping was then carried out for the detection of single-nucleotide polymorphisms in variants of the *GP130* (*IL6ST*) gene.

A cost-effective technique, T-ARMS-PCR, was preferred due to its allele specificity.¹⁵ Different specifications, conditions, and parameters for the T-ARMS-PCR primer design were considered using the default setting.

The *GP130* (*IL6ST*) gene sequence was retrieved by UniProt (<https://www.uniprot.org>). The entire nucleotide sequence of the *GP130* (*IL6ST*) gene was retrieved by the Ensemble Genome Browser 9.1.

The *GP130* (*IL6ST*) gene variants rs754547662 and rs747379809 were chosen for the detection of genetic polymorphisms from the Ensemble Genomic Browser: 9.1 (<https://asia.ensembl.org/index.html>). These variants were selected because of the presence of a severe consequence type: stop codon. The T-ARMS primers were adapted for rs754547662 and rs747379809 using Primer-1 (<https://primer1.soton.ac.uk/primer1.html>) and BLAST programme (<https://www.ncbi.nlm.nih.gov/blast>) to ensure precision (primer sequences are present in Appendix 1): rs754547662 primers: GF: G allele forward, TF: T allele forward, TR: T allele reverse, GR: G allele reverse; rs747379809 primer: TF: T allele forward, TR: T allele reverse, GF: G allele forward, GR: G allele reverse.

The optimised T-ARMS-PCR reaction for rs754547662 included 4 µL of Hot Start Taq 2x Master Mix (Cat. No. 901, Canada), 0.5 µL of each primer (10 pmol; Promega, USA), 1 µL of diluted gDNA, 0.6 µL of MgCl₂ (Thermo Fisher Scientific, USA, Cat. No. AB0359), 1 µL of reaction buffer (10x Taq Buffer; Cat. No. B55), and 1.4 µL of nuclease-free water (Sigma-Aldrich, molecular biology grade, Germany; Cat. No. W4502). All components were combined with a single Eppendorf tube. PCR was performed using a thermal cycler (Applied Biosystems, USA) under standard cycling conditions.

For rs747379809, the optimised mixture contained 6 µL of Hot Start Taq 2x Master Mix, 0.6 µL of each primer, 1.5 µL of

DNA, 1.3 µL of MgCl₂, 2 µL of buffer, and 1.8 µL of nuclease-free water. PCR products were analysed by 2% agarose gel electrophoresis stained with 5 µL of ethidium bromide in 50 mL 5x TAE buffer.

T-ARMS-PCR was used to amplify *GP130* gene variants rs754547662 and rs747379809 with allele-specific primers. For rs754547662, products included 385 bp (G allele), 426 bp (T allele), and 770 bp (internal control). For rs747379809, products were 263 bp (G), 311 bp (T), and 530 bp (control). Annealing temperatures were optimised at 63.0 °C and 59.3 °C for rs754547662 and rs747379809, respectively. For rs754547662, PCR began with an initial denaturation at 95 °C for 5 minutes, while for rs747379809, it was at 94 °C for 10 minutes. Both reactions underwent 30–35 cycles of denaturation, primer-specific annealing (63.0 °C for rs754547662 and 59.3 °C for rs747379809), and extension at 72 °C, followed by an extension at 72 °C for the final 10 minutes.

Alleles were identified by gel electrophoresis, and images were captured using the ChemiDoc Imaging System (Bio-Rad, USA). Genotypes were scored independently by a blinded analyst, and quality assurance was conducted to validate results.

Gold-standard Sanger DNA sequencing (Eurofins Genomics, Germany) was used to confirm the genotyping results of 30 samples. Only samples that met DNA quality control thresholds (integrity, concentration, and amplification clarity) were eligible, and from these, a random subset was chosen for sequencing. MEGA X (Molecular Evolutionary Genetics Analysis, version 10.2) was used for the analysis of sequencing results (Figure 1A and B).

All statistical analyses were conducted using SPSS version 23, with a significance threshold set at $p < 0.05$. An independent t-test was applied to compare the means of quantitative variables between the groups. Additionally, cross-tabulation was performed to calculate proportion percentages and examine relationships between categorical variables, such as gene polymorphisms and study groups.

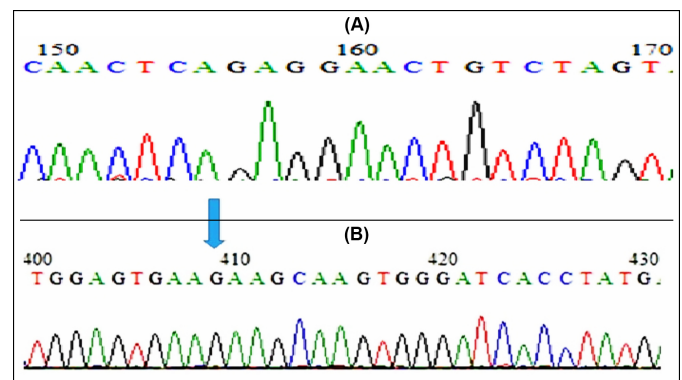


Figure 1: (A, B) Sequencing of the *GP130* gene variants: (A) rs747379809 and (B) rs754547662, showing the G allele.

Table I: Comparison of baseline characteristics between the UE-IF and fertile control groups.

Baseline characteristics	UE-IF Group Mean ± SD	Fertile Control Group Mean ± SD
Age of participant (years)	31.21 ± 5.72	31.08 ± 5.86
Age at menarche (years)	12.97 ± 0.96	13.17 ± 0.82
Infertility period (years)	8.06 ± 6.16	-
Duration of marriage (years)	8.74 ± 6.07	8.12 ± 4.78
Marital age (years)	22.53 ± 5.89	22.96 ± 4.32
Duration of menses (days)	4.58 ± 1.98	4.73 ± 1.09
Baseline characteristics	-	-
Haemoglobin (gm/dL) **	12.08 ± 1.16	11.38 ± 1.19
Haematocrit (%)**	43.17 ± 13.51	40.47 ± 9.68
Glycated haemoglobin (HbA1C) (%)**	4.79 ± 0.61	4.53 ± 0.59
Body mass index (BMI) Kg/m ² **	25.74 ± 5.22	24.73 ± 3.35
Systolic blood pressure (mmHg)	110.96 ± 13.09	111.53 ± 12.22
Diastolic blood pressure (mmHg)	78.3 ± 8.312	78.28 ± 8.03
Pulse (beats/minute)	78.95 ± 6.16	78.6 ± 5.52
Urea (mmol/L)	3.79 ± 1.54	3.92 ± 1.61
Sodium (Na ⁺), mEq/L**	139.53 ± 5.39	138.19 ± 5.19
Potassium (K), mEq/L	4.44 ± 0.78	4.39 ± 0.83
TSH mIU/L	3.61 ± 1.16	4.5 ± 1.52
FSH (IU/L)	5.78 ± 2.49	5.69 ± 2.54
Prolactin (ng/mL)	6.64 ± 3.49	6.64 ± 3.49
Body fat percentage** (%)	32.66 ± 6.21	31.42 ± 4.39

Values are presented as mean + SD and were computed by the independent t-test. TSH: Thyroid-stimulating hormone; FSH: Follicle-stimulating hormone.

** denotes statistically significant results ($p < 0.05$).

Table II: Genotype and allele frequencies of GP130 gene variants (rs747379809 and rs754547662) in the UE-IF cases and fertile control groups.

SNPs Genotypes and Alleles	rs747379809					rs754547662				
	GG	GT	TT	P(G)	P(T)	GG	GT	TT	P(G)	P(T)
UE-IF group	135 (100%)	0%	0%	270 (100%)	0 (0%)	135 (100%)	0%	0%	270 (100%)	0 (0%)
Controls group	177 (100%)	0%	0%	354 (100%)	0 (0%)	177 (100%)	0%	0%	354 (100%)	0 (0%)
Total	312 (100%)	0%	0%	624 (100%)	0 (0%)	312 (100%)	0%	0%	624 (100%)	0 (0%)

p-value is not applicable. Nucleotide base/allele: GT = Guanine and Thymine, GG = homozygous genotype, GT = heterozygous genotype, TT = homozygous genotype, rs = Reference SNP (Single nucleotide polymorphism).

RESULTS

A total of 312 subjects were divided into two groups based on fertility status: 135 (43%) females with UE-IF and 177 fertile controls (57%). Data are presented as mean ± SD (Table I).

The GP130 (*IL6ST*) gene variants rs747379809 and rs754547662 were categorised as homozygous dominant (TT), homozygous recessive (GG), and heterozygous variants (GT). In the present study population, only the ancestral G allele was detected across all participants (135 cases and 177 controls) for both variants. The T allele was absent. As a result, all individuals exhibited the homozygous wild-type genotype (GG), and no heterozygous (GT) or homozygous mutant (TT) genotypes were observed (Table II).

Due to this complete lack of allelic variation, the Hardy-Weinberg equilibrium (HWE) could not be assessed. A valid chi-square test under HWE assumptions requires the presence of all genotype categories, including heterozygous and mutant homozygous types. In the absence of such genetic diversity, HWE analysis is inapplicable because only one allele (the wild-type G allele) was present. Therefore, genotype frequencies were reported descriptively as percentages.

To validate the T-ARMS-PCR results, amplified and purified samples ($n = 30$) were subjected to gold-standard Sanger DNA sequencing (Figure 1A and B) to confirm the genotyping results and subsequently submitted to GenBank. The GenBank accession numbers are listed as: Seq1: *Homo sapiens* GP130 variant rs747379809 (PP782307); Seq2: *Homo sapiens* GP130 variant rs754547662 (PP782308).

DISCUSSION

Genetic analysis of rs754547662 (G/T) and rs747379809 (G/T) GP130 variants (consequence type: stop codon) revealed the presence of the major G allele and GG (homozygous) genotype in all cases and controls. In contrast, the mutant or risk-associated T allele, GT (heterozygous), and TT (Homozygous) were absent in both groups. The absence of risk allele T suggests that in women with UE-IF, no polymorphism was observed in the specific GP130 (*IL6ST*) gene variants rs74547662 (G/T) and rs747379809 (G/T).

Comparisons with prior studies show both similarities and population-specific differences. A study from Brazil investigating the GP130 (*IL6ST*) rs1900173 polymorphism in relation to fertility reported no significant association with female reproductive outcomes, aligning with the current

result.¹¹ In contrast, an Indonesian study supported the association between LIFR-GP130 receptor expression and successful reproductive outcomes in ART.¹⁶ However, scientists have discovered an association between UE-IF and polymorphism in the *LIFR* gene within the Pakistani population.¹⁷ A novel variant, rs121912501 C/T, has been identified as a stop-gained mutation that results in a truncated ligand-binding domain of LIFR.¹⁷ This structural alteration renders the receptor incapable of effectively binding its ligand LIF and is therefore considered a potential underlying genetic cause for UE-IF.¹⁷ LIFR rs121912501 TT and CT genotypes revealed an emerging threat for UE-IF.¹⁷ Subsequently, these findings may have a genetic basis related to the GP130 gene polymorphism, which may contribute to UE-IF.^{18,19}

For instance, a study conducted in Ohio, United States, revealed that mice with conditional deletion of uterine GP130 (*IL6ST*) or *Stat3* were associated with infertility,²⁰ and confirmed that both GP130 (*IL6ST*) and *STAT3* are critical for the fertility process.^{20,21} A Japanese study conducted in uterine LIFR-knockout and GP130 (*IL6ST*)-knockout mice models found that both models exhibited substantial downregulation of implantation-related genes and were completely infertile.²² Furthermore, decreased expression of LIFR and GP130 (*IL6ST*) has been witnessed in the endometrium of females with UE-IF in a Swedish study.²³ An Egyptian study reports that UE-IF women secreted significantly fewer GP130 (*IL6ST*) molecules in uterine flushing compared with fertile women, suggesting a potential functional difference.²⁴ These mechanistic studies highlight the biological, genetic, and physiological importance of the GP130 (*IL6ST*) gene and related signaling for the successful outcome of pregnancy.

Despite the biological coherence suggested by experimental literature, the present results indicate that the specific GP130 (*IL6ST*) variants rs75454766 (G/T) and rs747379809 (G/T) do not contribute to UE-IF in this study population. The absence of the T allele in both groups may indicate a lack of variability, potentially due to population homogeneity rather than a lack of biological relevance.

This study is the first effort in Pakistan to explore the potential pathophysiological role of GP130 (*IL6ST*) gene variants in women with UE-IF, particularly in a resource-limited setting where empirical management is not feasible. Despite employing high-throughput T-ARMS-PCR and a robust sample size, no association was found between UE-IF and two investigated SNPs of the GP130 (*IL6ST*) gene. However, this finding serves as a foundational step toward identifying relevant genetic markers.

Expanding genotyping to additional GP130 (*IL6ST*) variants may further clarify genetic risks associated with UE-IF and support informed, evidence-based reproductive care. The small sample size, absence of gene expression analyses, and uninvestigated JACK-STAT pathway were limitations of the study.

CONCLUSION

In women with UE-IF, no polymorphism was observed in the specific GP130 (*IL6ST*) gene variants rs74547662 (G/T) and rs747379809 (G/T). Hence, the GP130 (*IL6ST*) gene polymorphism (rs74547662 and rs747379809) is not associated with UE-IF in this study population. The presence of the major G allele and the absence of the minor/risk T allele in all cases and controls indicates population homogeneity or limited genetic and ethnic diversity, rather than a lack of biological relevance.

Furthermore, these results suggest that variations in this signalling pathway do not contribute significantly to the aetiology of UE-IF in the studied population. Further research involving larger cohorts and additional molecular markers may help clarify the complex genetic landscape underlying this condition.

The analysis of additional variants of the GP130 (*IL6ST*) gene with an increased sample size and greater ethnic diversity will provide more comprehensive insights into GP130 (*IL6ST*) gene polymorphisms in UE-IF.

ETHICAL APPROVAL:

Ethical approval was obtained from the Institutional Bioethics Review Committee of the University of Karachi, Karachi, Pakistan (Approval No. IBC124-B/2020).

PATIENTS' CONSENT:

Informed consent was obtained from all participants.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

RA, RR: Conception and design of the study, acquisition, analysis, interpretation of data, and drafting of the manuscript for important intellectual content.

HNK, MUNI: Data analysis, data interpretation, and critical review of the manuscript.

TAK: Critical revision of the manuscript for important intellectual content.

All authors approved the final version of the manuscript to be published.

Appendix 1: Primers for the GP130 gene variants rs754547662 and rs747379809.

SNPs	Primers	Primer sequence	Annealing temperature	Product size
rs754547662	GF	5'- CTGGAGTGACTGGAGTGGAG-3'	58°C	G allele: 385 T allele: 426 IC: 770
	TR	5'- ATAGGTGATCCCACTTGCCTA -3'		
	TF	5'- ACATGGTGAATCTTGTGATACTAAA -3'		
	GR	5'- TAATAGAGAAGGGGTTTCACCATAT -3'		
rs747379809	TF	5'- TCAGTGATCAACTCAGGGT -3'	53°C	T allele: 311 G allele: 263 IC: 530
	GR	5'- TCAATTTTAAGATACTAGACAGCTC -3'		
	GF	5'- GAACTGAAGATTCTTTAGAAATCTT -3'		
	TR	5'- GTATTTCTATCTGGATATCTTCT -3'		

rs754547662 Primers: GF: G allele forward; TF: T allele forward; TR: T allele reverse; GR: G allele reverse.

rs747379809 Primer: TF: T allele forward; TR: T allele reverse; GF: G allele forward; GR: G allele reverse. IC: Internal control.

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