ISSN 2277-8330 *(Electronic)*

*J.Bio.Innov2(3)*,pp:79-89,2013

www.jbino.com

# DEVELOPMENT AND VALIDATION OF A PCR-RFLP METHOD TO GENOTYPE THE *CRHR1* (rs242941: G>T)) GENE VARIATION: A SIMPLE AND INEXPENSIVE PHARMACOGENETIC TOOL

**Neeraj Sharma[1], Dr. Shally Awasthi[1*], Dr. Subha R Phadke[2], Dr. Sarika Gupta[1] &**

**Dr. Sarita Agrawal[2]**

[1]Department of Paediatrics & Translational Medicine Unit, KGMU, Lucknow-UP, India.
[2]Department of Medical Genetics, SGPGIMS, Lucknow –UP, India.

**Email**: shallya@rediffmail.com

(Received on Date: 2nd March 2013 Date of Acceptance: 8th April 2013)

## ABSTRACT

Corticotrophin releasing hormone receptor1 (CRHR1) is a potent mediator of endocrine, autonomic, behavioural, and immune responses to stress, supposed to play a pivotal role in steroid pathway. The genetic variations of this gene have significant influences in response to corticosteroid therapy in a wide range of disease. Number of genotyping methods has been developed to investigate the genetic variants of this gene. However, classical polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis is still lacking. Therefore, we aimed to develop this straightforward and affordable method to detect the nucleotide variation (rs242941: G>T) of CRHR1gene, can apply in basic research study of complex genetic diseases. The 100 clinically defined asthmatic patients from North India region were recruited for this study and their DNA were extracted. Primer set was designed by Batch primer3 Software. The PCR-RFLP assays were performed by endonuclease (AciI) digestion of PCR-amplified DNA visualized in agarose gel. The allele frequencies for G>T variation were 0.74 (G allele) and 0.25 (TT allele). This work is the first to provide evidence for PCR-RFLP being the method of choice for CRHR1:rs242941 SNP genotyping. This is affordable, specific, reproducible, with sufficiently throughput capacity and particularly appropriate for medium- scale genotyping purposes.

***Keywords:*** *Genetic testing, Mutation detection, Genotyping, Single nucleotide polymorphisms, Genotyping method*

| **Number of Tables: 3** | **Number of Figures: 3** | **Number of References: 25** |
|---|---|---|

## INTRODUCTION

The majority of genetic variations (>90%) in the human genome are single nucleotide polymorphisms which might be responsible for inter-individual response to drug. These variations are commonly inherited and pose substantial clinical problems (Collins *et al*., 1998; Pang *et al*., 2009). Over the last two decades, several techniques have been developed for genotyping of SNPs found in candidate genes involved with complex genetic diseases. Some of them are differential hybridization based, allele-specific genotyping, primer extension, oligonucleotide ligation, DNA sequencing and application of endonuclease cleavage for allele discrimination (Chatterjee *et al*., 1999; Iwasaki *et al*., 2002; O'Meara *et al*., 2002; Papp *et al*., 2003). All of the above mentioned methods require simple and easily available thermo cycler machine.

Recent developments in technology for SNP genotyping (by Taqman method, Invader method, MALDI-TOF method, Gene Chips) not only improve the output but also improve the quality of result in very less time (Livak *et al*., 1995; Haff *et al*., 1997; Kwiatkowski *et al*., 1999; Gunderson *et al*., 2005). The major drawback of these techniques is the requirement of expensive equipment and well trained hands. However, PCR-RFLP is still an inexpensive, simple and convenient technique for genotyping of SNPs. It is being widely used in modern molecular genetic studies with medium throughout facilities.

PCR-RFLP, also known as cleaved amplified polymorphic sequences (CAPS) is based on generation and deletion of cleavage site for specific restriction endonuclease by nucleotide variations in the PCR-amplified DNA. The type of SNP can easily be identified by size discrimination of the digested amplified DNA, during gel-electrophoresis. It is a simple, sensitive and reliable method requires minimal expenditure in instrumentation (Ota *et al*., 2007).

The corticotropin–releasing hormone (CRH) is a well-known neuroendocrine mediator of behavioural and immune response to stress. It is released from the hypothalamus upon exposure to stressful signals and binds to the *CRHR1* (Bittencourt e*t al*., 2000; Treutlein *et al*., 2006). *CRHR1* is a predominant receptor in pituitary gland, regulates the adreno-corticotropic hormone (ACTH) and the catecholaminergic response to CRH. Alteration of any of the CRH effects as mediated by the CRHR1 (NM_004382, 17q12q22) can influence the adminis-tration of corticosteroid drugs as it was evidenced by Tantisira *et al.*, 2004, in asthmatic patients. Therefore a relation is expected to exist between genetic variation in *CRHR1* gene and endogenous cortisol secretion, as well as administered exogenous corticosteroids. A significant association between 8-week FEV1 response to ICs and variation of *CRHR1* gene have also been observed in both adult and paediatric asthmatic patients. The association of rs242941 with positive treatment response (about two and half times improvement in FEV1) has been observed in both adults (P=0.025) and Childhood Asthma Management Program (CAMP) populations (P=0.006) (Tantisira *et al*., 2004; Lima *et al*., 2009). The published data indicate that genetic

variations in *CRHR1* (rs242941; chr17:41248300) have pharmacogenetic effects influencing asthmatic response to corticosteroids; suggest that this gene pathway can be a novel therapeutic target in asthmatics (Sun *et al*., 2000; Tantisira *et al*., 2004; Lima *et al*., 2009; Sharma *et al*., 2012).

Previously, genotyping for SNP (rs242941: G>T) was carried out by both direct sequencing and SEQUENOM Mass ARRAY MALDI-TOF mass spectrometer (Sun *et al*., 2000; Kim *et al*., 2009). However, the use of this advance technology is still costly for routine application. Therefore, there is need to develop a reliable and inexpensive genotyping method, which can useful in various pharmacogenetic and epidemiological studies. We aim to undertake this task by developing novel PCR-RFLP method for *CRHR1* (rs242941: G>T) gene polymorphism.

## MATERIALS AND METHODS

### Study subjects and DNA extraction

We selected 100 North-Indian children with clinically diagnosed asthma. The diagnosis of asthma was based on physician's assessment. A written informed consent was signed by the parents of all the subjects. The study was approved by the Institutional ethical committee. Blood (1mL) was collected in EDTA tube from the subjects. Genomic DNA was isolated from blood samples using a well-known salting out method (Miller *et al*., 1988). The purity of template DNA is determined by calculating the ratio of absorbance at 260nm and 280nm.The template DNA having an $A_{260}/A_{280}$ ratio of 1.7-1.9 was taken for PCR procedure.

### Design of PCR primers and Selection of restriction endonuclease

To obtain high quality result through PCR-RFLP, the designing of specific primers and selection of appropriate restriction enzyme is the most important part of the assay development. To assist this tedious step a web based automated primer designing tool Batch primer3 (accessible at http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) has been used to design generic oligoes (Ye *et al.*, 2001),(Table1). To overcome the amplification of an orthologous nonspecific region or paralogous region, the selected primers pair was validated against the BLAST database (accessible at http://blast.ncbi.nlm.nih.gov/Blast.cgi). A hypothetical sequence generated from the designed primer was obtained from the UCSC Genome Bioinformatics server by using "UCSC In-Silico PCR" (accessible at http://genome.ucsc.edu/cgi-bin/hgPcr?command=start), figure 1. The selection of restriction endonuclease "*AciI*" and the size of digested PCR fragments were determined by NEBcutterV2.0 (a comprehensive web-based freely available tool, accessible at http://tools.neb.com/NEBcutter2/ ), figure 2.

### PCR setup

PCR was carried out in a total volume of 10µl reaction mixture, with 30ng of genomic DNA, 1pmol of each primer, 1µl 10X buffer, 1.5mM $MgCl_2$, 200µM dNTPs and 0.5U Taq polymerase *(NEW ENGLAND Biolabs$_{INC}$*, England). The PCR cycle conditions are shown in table 2.

The three hundred eighty one base pair PCR products were visualized in an ethidium bromide (EtBr) stained 2% (wt/vol) agarose gel in 1X TBE buffer, gel was loaded by 100bp DNA size marker in separate lane that was parallel to the sample.

**Endonuclease Restriction Assay**

10µl of RFLP mixture was prepared in a 0.2ml PCR tube and incubated at 37°C for overnight (16h). The RFLP mixture consisted of 5µl PCR product, 1X buffer and 1U *AciI* (*NEW ENGLAND Biolabs*$_{INC}$, England) restriction enzyme. The RFLP reaction mixture was gently tapped and centrifuged for five second before incubation in Digital dry Bath.

**Genotyping and agarose gel electrophoresis**

Overnight incubated PCR products were separated on 2% agarose gel, prepared and immersed in to an electrophoresis gel chamber containing 1X TBE buffer. The 5µl digested samples with 1X gel loading dye were loaded into the wells and the system was run at 200 volt for 45 minute. The expected size for specific allele (GG=225bp+156bp; GT=381bp+225bp+156bp; TT=381 bp) in digested PCR products were visualised (figure 3a) on UV-transilluminator-DUAL (Medox-Bio$^{TM}$).

**Controls**

A study sample was used as control (negative control: without template and positive control: with template) during PCR in parallel with other test samples but in different tubes. Prior to its use as control the PCR product length was confirmed by DNA molecular marker and presence of SNP site was confirmed by sequencing. The amplification product of this control sample was also included during the run of each gel electrophoresis to confirm the enzyme activity in different tubes.

**Validation of PCR-RFLP results**

10% randomly selected samples were sequenced by outsource using an Applied Bio-systems 3730 DNA analyser and ABI-Biosciences sequence analysis software (Eurofins Genomics India Pvt, Bangalore).The reproducibility of the PCR-RFLP method was assessed by repeating the genotyping of SNP for 50% samples in an independent experiment.

**RESULTS & DISCUSSION**

All the 100 subjects were successfully genotyped in our studied population. The frequency of each allele was 74.5% for allele G and 25.5% for allele T, giving an estimated frequency of 55%, 39% and 6% respectively for genotype GG, GT and TT (Table 3). 100% concordance has been seen between results of PCR-RFLP genotyping and sequencing (figure 3b).

**Discussion**

In the modern genetic era, various reliable and accurate molecular genotyping methods are available. Still, search for easy, accurate and cost effective technique continues for laboratories not equipped with high throughput screening equipment. Relatively, PCR-RFLP is a simple, convenient and inexpensive laboratory technique. It is especially useful in small scale research studies of complex genetic disease and related SNPs (Zhang *et al*., 2005; Ota *et al*., 2007; Yang *et al*., 2010).

We first designed the primer set in such a way that it avoids repetition of selected restriction endonuclease site (*AciI*) in the PCR fragment. The obtained digested fragments are easy to discriminate on agarose gel electrophoresis. To eliminate non-specific PCR product, we selected a specific annealing temperature after a gradient PCR experiment. This annealing temperature ensures the specific and efficient binding with template DNA and produced three hundred eighty one base pair PCR products in the reaction mixture. A sample was used as control during PCR and restriction digestion experiment to avoid genotyping error due to undigested PCR products.

This is first study of its kind to determine genotype and allele frequency of SNP rs242941: G>T by PCR-RFLP method in North Indian population. This method was successfully used for the genotyping of 100 clinically defined asthmatics. The concordant sequencing results of randomly selected 10% sample with PCR-RFLP results support the reliability of this conventional method.

In conclusion, we have developed a validated and simple PCR-RFLP method for genotyping of SNP in *CRHR1* (rs242941) gene which is more cost effective than sequencing and other complex typing methods.

**ACKNOWLEDGEMENTS**

The authors are grateful to Dr. A.A. Mehdi (Prof & Head), Department of Biochemistry, KGMU, Lucknow, UP, India, for providing instrument facility. This work was supported by the CST-UP grant CST/SERPD/D-1153, 4, August 2011, UttarPradesh,India.

***Table 1:*** *Primers used for PCR amplification of rs242941 and their concentration in each reaction mixture.*

| Primers | Sequences (5’ -> 3’) | Tm$^{o}$ C | Primer Conc. (pMol) |
|---|---|---|---|
| Forward Primer (F) | GACACTTCAGGAGGGGAGGGTGGATATG | 69.5 | 1 |
| Reverse Primer (R) | CTGAGTCCAGCAGAGAAAGGGAGCCAAT | 68.0 | 1 |

***Table 2:*** *Polymerase Chain Reaction cycle conditions*

| PCR Cycle steps | Temperature ( °C) | Time (s) |
|---|---|---|
| Initial activating step | 95 | 120 |
| 3-step cycling | | |
| Denaturation | 95 | 60 |
| Annealing | 64 | 45 |
| | 72 | 60 |

| | | |
|---|---|---|
| Extension | | 35 |
| Number of cycles | 72 | 120 |
| Final extension | | |

***Table 3:*** *Genotype and allele frequency for SNP (CRHR1; rs242941)*

| Genotype | Frequency% |
|---|---|
| **GG** | 55(55.0%) |
| **GT** | 39(39.0%) |
| **TT** | 6(6.0%) |
| **Allele** | |
| **G** | 149(74.5%) |
| **T** | 51(25.5%) |

***Fig 1:*** *Schematic presentation of the CRHR1 gene and SNP (rs242941; chr17:43892520). Exons and introns are represented by cylinders and lines, respectively. The UCSC In-Silico PCR sequence along with restriction site showed between the arrows. The primer sequences are highlighted in green colour. Arrow '▲' represents SNP site in the PCR product.*

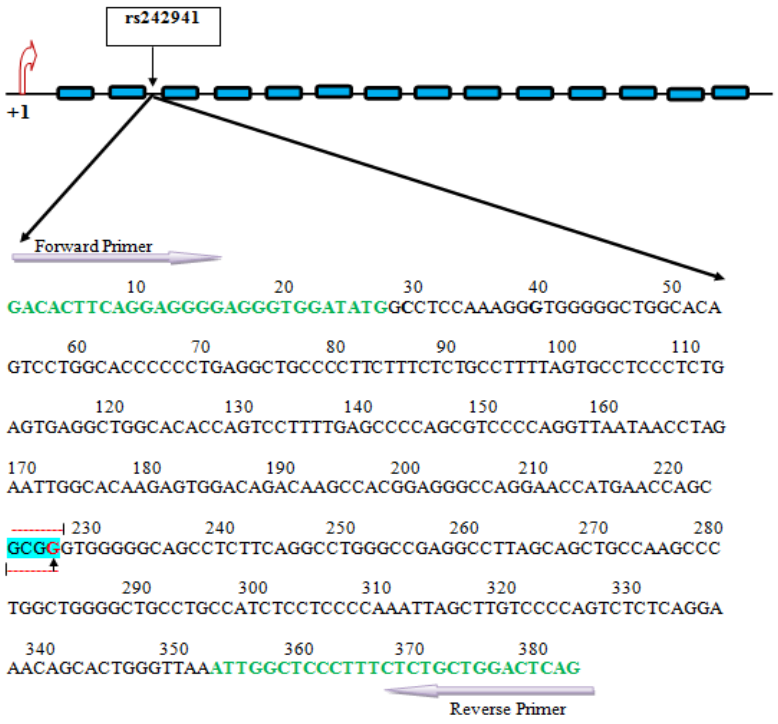


***Fig 2:*** *A diagrammatic representation of PCR-RFLP principle used to genotype the CRHR1 (rs242941: G>T) variation.*

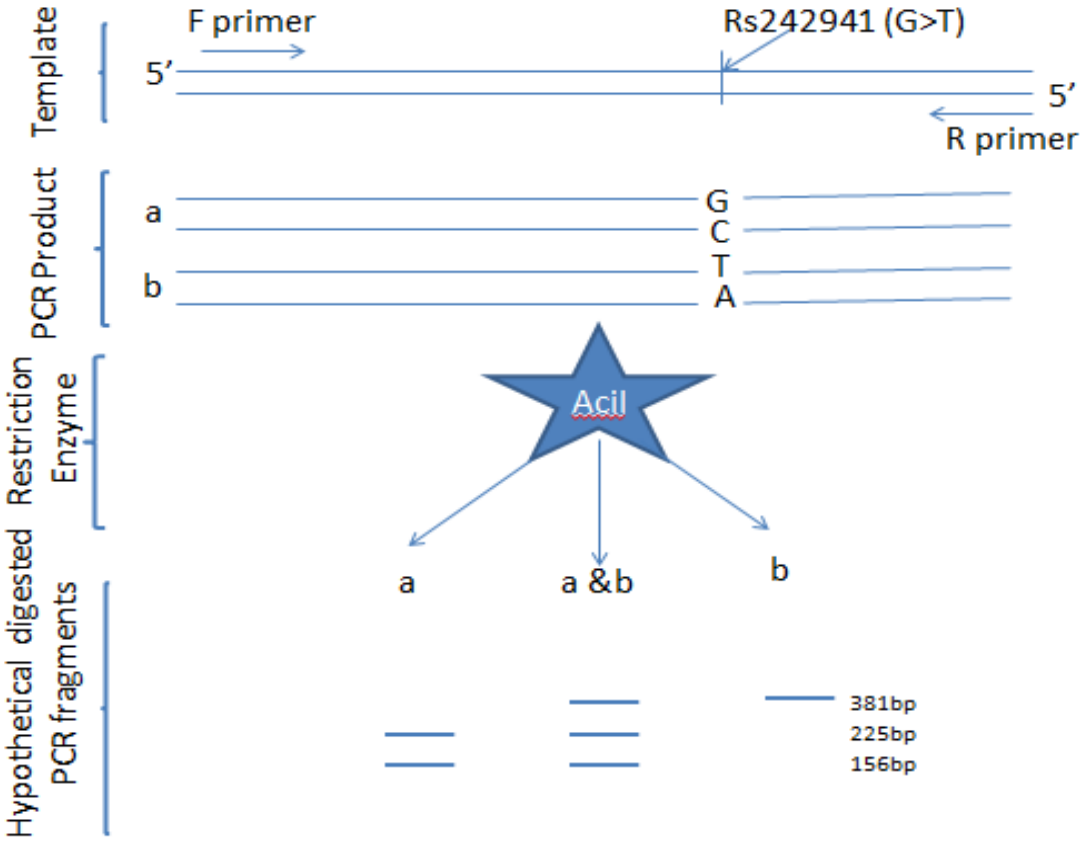
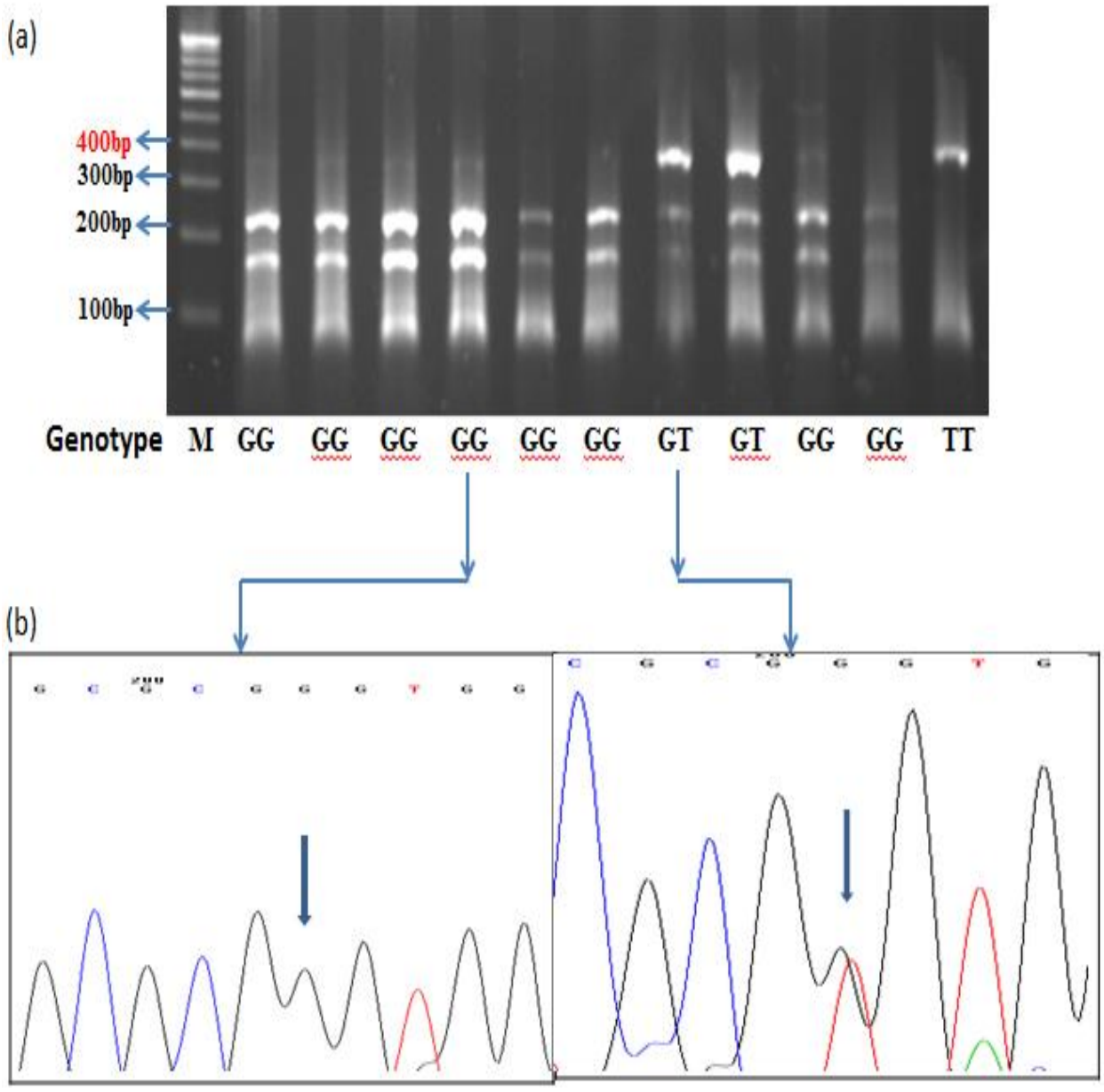


***Fig 3:*** *Genotyping of SNP (rs242941) by PCR-RFLP. (a) Agarose gel Electrophoresis showing M, 100bp molecular marker; GG, 225bp&156bp; GT, 381bp, 225bp&156bp; TT, 381bp.( b) PCR- Sequencing Chromatograms for GG and GT samples respectively.*



## REFERENCES

**Bittencourt, J.C. and Sawchenko, P.E.** (2000) Do centrally administered neuropeptides access cognate receptors? an analysis in the central corticotropin-releasing factor system. *J Neurosci* **20**:1142-1156.

**Collins, F.S., Brooks, L.D., and Chakravarti, A.** (1998) A DNA polymorphism discovery resource for research on human genetic variation. *Genome Res* **8**:1229-1231.

**Chatterjee, P.K., Yarnall, D.P., Haneline, S.A., Godlevski, M.M., Thornber, S.J., Robinson, P.S., Davies, H.E., White, N.J., Riley, J.H. and Shepherd, N.S.** (1999) Direct sequencing of bacterial and P1 artificial chromosome-nested deletions for identifying position-specific single-nucleotide polymorphisms. *Proc Nat Acad Sci* **96:**13276-13281.

**Gunderson, K.L., Steemers, F.J., Lee, G., Mendoza, L.G. and Chee M.S.** (2005) A genome-wide scalable SNP genotyping assay using microarray technology. *Nat Genet* **37**:549-554.

**Haff, L.A. and Smirnov, I.P.** (1997) Single-nucleotide polymorphism identification assays using a thermostable DNA polymerase and delayed extraction MALDI-TOF mass spectrometry. *Genome Res* **7**:378-388.

**Iwasaki, H., Ezura, Y., Ishida, R., Ishida R., Kajita, M., Kodaira, M., Knight,J., Daniel, S., Shi, M. and Emil, M.** (2002) Accuracy of genotyping for single nucleotide polymorphisms by a microarray -based single nucleotide polymorphism typing method involving hybridization of short allele-specific oligonucleotides. *DNA Res* **9**:59-62.

**Kwiatkowski, R.W., Lyamichev, V., de Arruda, M. and Neri, B.** (1999) Clinical, genetic and pharmacogenetic applications of the Invader assay. *Mol Diagn* **4**:353-36

**Kim, W.J., Sheen, S.S., Kim, T.H., Huh, J.W., Lee, J.H., Kim, E.K., Lee, J.H., Lee, S.M., Lee, S., Lim, S.Y., Shin, T.R., Yoon, H.I., Oh, Y.M. and Lee, S.D.** (2009) Association between CRHR1 polymorphism and improved lung function in response to inhaled corticosteroid in patients with COPD. *Respirology* **14**:260-263.

**Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W. and Deetz, K.** (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* **4:**357-362.

**Lima, J.J., Blake, K.V., Tantisira, K.G. and Weiss, S.T.** (2009) Pharmacogenetics of asthma. *Curr Opin Pulm Med* **15**:57-62.

**Mullis, K.B. and Faloona, F.A.** (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol* **155**:335-350.

**Ota, M., Fukushima, H., Kulski, J.K. and Inoko, H.** (2007) Single nucleotide polymorphism detection by polymerase chain reaction-restriction fragment length polymorphism. *Nat Protoc* **2**:2857-2864.

**O'Meara, D., Ahmadian, A., Odeberg, J. and Lundeberg, J.** (2002) SNP typing by apyrase-mediated allele-specific primer

extension on DNA microarrays. *Nucleic Acids Res.* **30**:e75.

**Miller, S.A., Dykes, D.D. and Polesky, H.F.** (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**:1215.

**Pang, G.S., Wang, J., Wang, Z., Lee C.G.** (2009) Predicting potentially functional SNPs in drug-response genes. *Pharmacogenomics* **10**:639-653.

**Pickering, J., Bamford, A., Godbole, V., Briggs, J., Scozzafava, G., Roe, P., Wheeler, C., Ghouze, F. and Cuss, S.** (2002) Integration of DNA ligation and rolling circle amplification for the homogeneous, end-point detection of single nucleotide polymorphisms. *Nucleic Acids Res* **30**:e60.

**Papp, A.C., Pinsonneault, J.K., Cooke, G. and Sadee, W.** (2003) Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves. *Biotechniques* **34:**1068-1072.

**Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N.** (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.

**Sharma, N., Awasthi, S., Phadke, S.R. and Gupta, S.** (2012) Genotyping the *CRHR1* rs242939 (A > G) Polymorphism by a One-Step Tetra Primer–Amplification Refractory Mutation System–Polymerase Chain Reaction. *Genet Test* **16**:794-797.

**Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B. and Erlich, H.A.** (1986) Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature* **324**:163-166.

**Ye, S., Dhillon, S., Ke, X., Collins, A.R, and Day, I.N.** (2001) An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res* **29**: e88.

**Sun, X., Ding, H., Hung, K. and Guo B.** (2000) A new MALDI-TOF based mini-sequencing assay for genotyping of SNPS. *Nucleic Acids Res* **28**: E68.

**Treutlein, J., Kissling, C., Frank, J., Wiemann, S., Dong, L., Depner, M., Saam, C., Lascorz., J, Soyka, M., Preuss, U.W., Rujescu, D., Skowronek, M.H., Rietschel, M., Spanagel, R., Heinz, A., Laucht, M., Mann, K. and Schumann, G.** (2006) Genetic association of the human corticotropin releasing hormone receptor 1 (*CRHR1*) with binge drinking and alcohol intake patterns in two independent samples. *Mol Psychiatry* **11**:594-602.

**Tantisira, K.G., Lake, S., Silverman, E.S., Palmer, L.Z., Lazarus, R, Silverman, E.K. Liggett, S.B., Gelfand, E.W., Rosenwasser, L.J., Richter, B., Israel, E., Wechsler, M., Gabriel, S., Altshuler, D., Lander, E., Drazen, J. and Weiss, S.T.** (2004) Corticosteroid pharmacogenetics: association of sequence variants in *CRHR1* with improved lung function in asthmatics treated with inhaled corticosteroids. *Hum Mol Genet* **13**:1353-1359.

**Zhang, R., Zhu, Z., Zhu, H., Nguyen T, Yao, F., Xia, K., Liang, D. and Liu, C.** (2005) SNP cutter: a comprehensive tool for SNP PCR-RFLP assay design. *Nucleic acid Res* **33**:489-492.