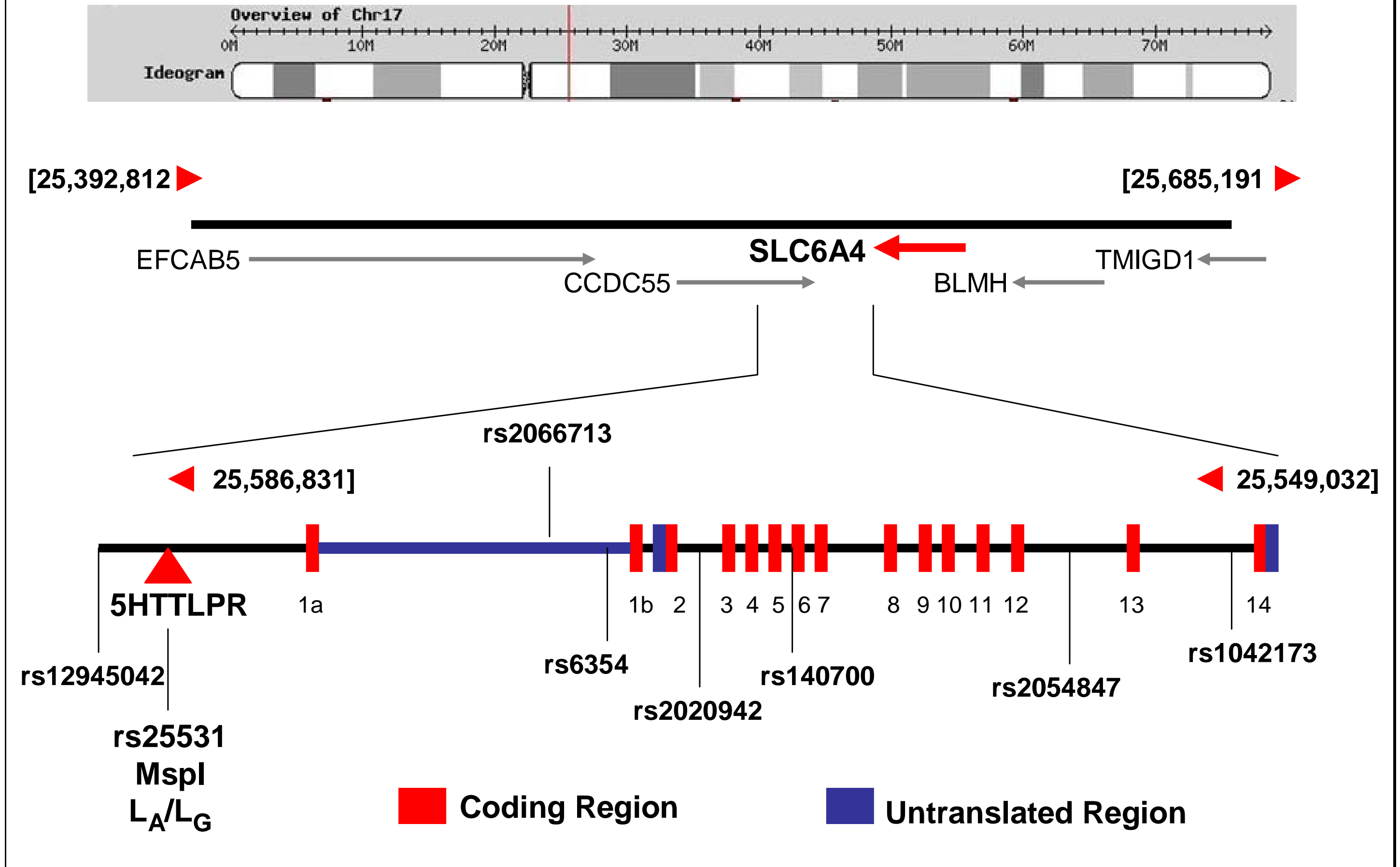
Serotonin Transporter-Linked Polymorphic Region (5HTTLPR)
and
rs25531 SNP (MspI, L_A / L_G)

Serotonin Transporter (5HTT, Locus Symbol SLC6A4), which maps to 17q11.1-17q12 (Ramamoorthy et al., 1993), contains a **43 bp insertion/deletion** (ins/del, **5HTTLPR**) polymorphism in the 5' regulatory region of the gene (Heils *et al.,* 1996). It should be noted that due to an error in sequencing this was originally thought to be a 44 bp deletion (the highly repetitive nature of this site makes this a very excusable error). The ins/del in the promoter appears to be associated with variations in transcriptional activity: the long variant (**L**) has approximately three times the basal activity of the short promoter (**S**) with the deletion (Lesch *et al.,* 1996), although this is not a universal finding (Willeit et al., 2001, Kaiser et al., 2002). The **S** variant has been reported to be dominant over the **L** variant (Heils et al., 1996), although at least one report suggests that the **L** may be dominant over the **S** (Williams et al, 2003). Several investigators have reported that the 5-HTTLPR polymorphism affects serotonergic functions in vivo. Individuals with the **L/L** genotype were found to have significantly higher maximal uptake of serotonin into platelets compared to those with **L/S** or **S/S** genotypes (Nobile et al., 1999, Greenberg et al., 1999).



A depiction of the organization of the serotonin transporter showing the 5HTTLPR region and the positions of several SNPs that will be used in other analyses.
Adapted from Heils et al, 1996 and Lesch et al, 1996.

The assay we use for the 5HTTLPR (Anchordoquy et al, 2003) is a modification of the method of Lesh *et al*, (1996). The primer sequences are from Gelernter et al. (1999).

Forward: 5'- 6FAM - ATG CCA GCA CCT AAC CCC TAA TGT - 3',
Reverse: 5'- GGA CCG CAA GGT GGG CGG GA - 3'.

These primers yield amplicons of 419 (**L**) or 376 (**S**) bp.

5HTTLPR PCR Master Mix for 20 µL reactions

(18 µL Master mix + 2 µL DNA)

Component	1 Tube vol (µL)	100 Tubes vol (µL)	Concentration of component in: Stock	Master Mix	PCR
Water	9.3	930			
DMSO	2.0	200	100%	10.9 %	10%
10x Buffer II	2.0	200	10 x	0.109 x	1 x
$MgCl_2$	1.6	160	25 mM	2.18mM	2.00 mM
dNTP+deazaGTP	2.0	200	2 mM (ea)	218 µM	200 µM (ea)
Forward	0.65	65	12 µM	425 µM	380 µM
Reverse	0.65	65	12	425 µM	380 µM
AmpliTaq Gold®	0.2	20	5 units/µL	1 unit	1.0 unit
Total volume (µL)	18.4	1840			

Preparation of dNTPs + 7-deaza-2-deoxy GTP

Component	volume (µL)	Concentration (mM) Stock	Final
dATP	40	100	2
dTTP	40	100	2
dCTP	40	100	2
dGTP	20	100	1
deazaGTP	200	10	1
Water	1660		

5HTTLPR PCR Setup

Mastermix	18 µL
DNA	1-2 µL (20 ng or less)
Water	0-1 µL
Total volume	20 µL

5HTTLPR Touchdown PCR Cycling

1x	95 °C	10 min				
1x	95 °C	30 sec	65 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	64 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	63 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	62 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	61 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	90 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	59 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	58 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	57 °C	30 sec	72 °C	90 sec
1x	95 °C	30 sec	56 °C	30 sec	72 °C	90 sec
30x	90 °C	30 sec	55 °C	30 sec	72 °C	90 sec
1x	72 °C	30 min				
	4 °C	hold				

5HTTLPR Electrophoresis

2 µL PCR product
20 µL Hi-Di formamide
0.5 µL Genescan 500 (or 2500) Rox

Samples are analyzed on an ABI PRISM® 3130xl Genetic Analyzer using standard company protocols without modification

5HTTLPR. The figure above is reproduced from a run from an ABI PRISM® 3100 Genetic Analyzer. The amplicons are labeled with size in base pairs. The sizes given are those calculated from the DNA sequence, but in actual runs the size of the amplicon sizes calculated by the software are usually 3-4 bp greater. The figure above shows both alleles which generally have approximately equal peak heights. The red peaks are size standards (Genescan ROX 500).

The table below lists the frequencies of the two alleles in approximately 1000 subjects taken from the National Youth Survey Family Study. These are typical for a largely Caucasian population such as this.

Amplicon Size	Allele	Frequency

376	short	.43
419	long	.57

Notes:

For consistent results with this primer set the use of 10% DMSO and 7-deaza-2-deoxy GTP (Roche Applied Science, Indianapolis, IN) is essential.

Use a very good grade of DMSO. We use Sigma's Hybra-Max® grade or that supplied with New England Biolab's Phusion™ buffers.

We use touchdown PCR (Don et al, 1992) routinely as a simple short cut. It cuts down on the need to optimize annealing conditions for multiple primer sets when you want to do several loci in the same thermocycler.

There are many papers on this polymorphism, which can lead to confusion at first. Depending on the primer sets used and the nomenclature the authors use, the sizes of the reported long and short alleles may be different. Ours, using the primers reported by Gelernter et al (1999) are 376 and 419 bp. Heils et al (1996) report 484 and 528 bp; Wendland et al (2006) report 469 and 512 bp; and Nakamura et al (2000) refer to them as 14- and 16-repeat alleles. These are all the same.

rs25531 SNP (MspI, L_A / L_G)

Hu et al (2006) reported that a SNP (rs25531, A/G) in the Long form of 5HTTLPR may have functional significance: The more common L_A allele is associated with the reported higher basal activity, whereas the less common L_G allele has transcriptional activity no greater than the S. These investigators suggest that in tests of association the L_G alleles should be analyzed along with the S alleles.

The substitution of the G for A in the SNP, produces and MspI restriction site (CCGG) which forms the basis of the analysis strategy (Wendland et al, 2006). The sequence of the 5HTTLPR region was first reported by Heils, et al, and is reproduced below in the way they did to show the highly repetitive nature of the locus. There were two errors in their sequence in repeat units III and V (underlined) that have been corrected here. The forward and reverse primers we use now that yield amplicons of 376 and 419 bp, are shown in yellow highlight. The SNP, rs25531 is shown as "R" in green highlight and the second MspI site in repeat unit XIV is shown in teal highlight. The insertion/deletion is shown underlined in lower case in repeat units VI, VII, and VIII.

TCTCCCGCCTGGCGTTGCCGCTCTGAATGCCAGCACCTAACCCCTAATGT

I   CCCTAC TGCA GCCCCCCC AGCAT

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II    CCCCCC TGCA ACCTCCC  AGCA
III   ACTCCC TGTA CCCCTCCT AGGAT
IV    CGCTCC TGCA TCCCCC   ATTATC
V     CCCCCC TTCA CCCCTCGC GGCAT
VI    CCCCCC TGCA ccccc    Rgcat     R = A or G
VII   cccccc tgca gccccccc agcat
VIII  ctcccc tgca CCCCC    AGCAT
IX    CCCCCC TGCA GCCCTTCC AGCA
X     TCCCCC TGCA CCTCTCCC AGGAT
XI    CTCCCC TGCA ACCCCC   ATTAT
XII   CCCCCC TGCA CCCCTCGC AGTAT
XIII  CCCCCC TGCA CCCCCC   AGCATC
XIV   CCCCCA TGCA CCCCC    GGCAT
XV    CCCCCC TGCA CCCCTCC  AGCAT
XVI   TCTCCT TGCA CCCTACC  AGTAT

      TCCCCCGCATCCCGGCCTCCAAGCCTCCCGCCCACCTTGCGGTCCCCGCC
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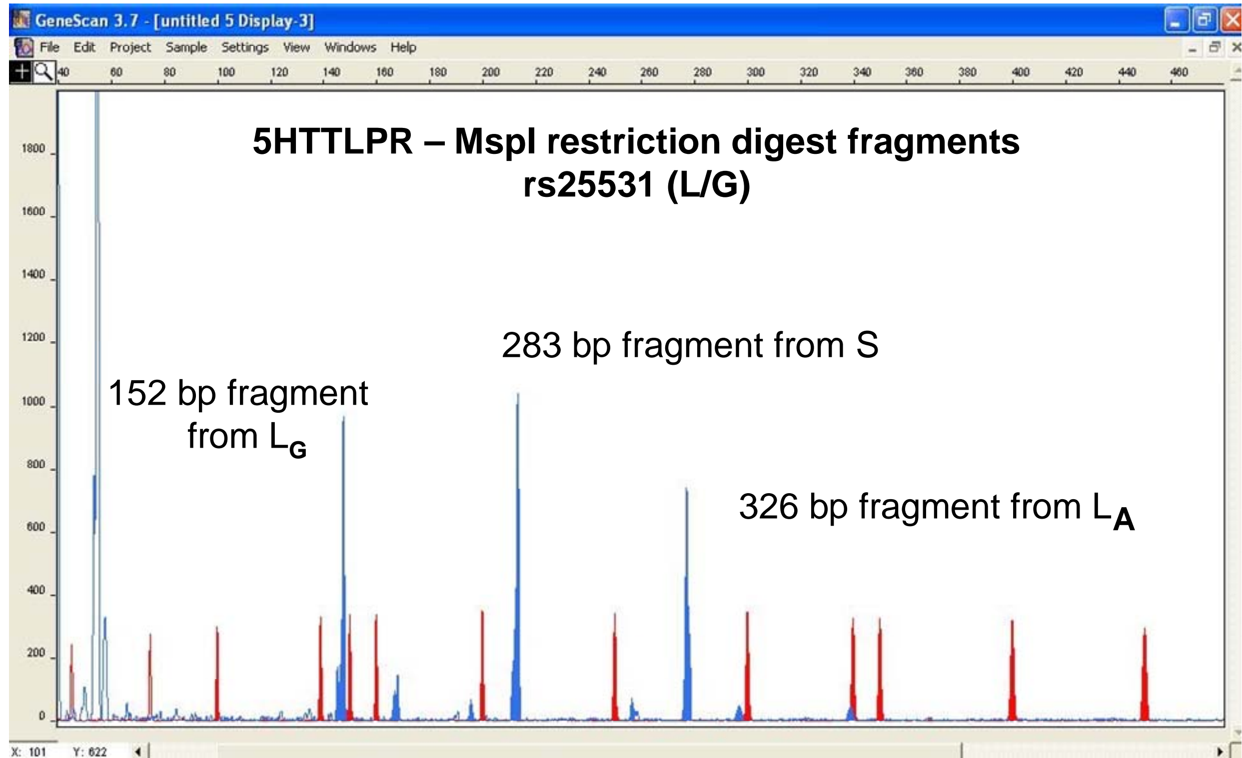
The forward primer has a fluorescent label attached to it's 5' end. To analyze this SNP, the full length amplicons (from above) are incubated with the restriction enzyme MspI. The G allele which has the MspI restriction site (CCGG) will yield a product of 152 bp, whereas the A allele, which lacks the restriction site does not. A second MspI site 93 bp from the 3' end of the amplicon provides a positive control for the restriction reaction yielding cut products of 326 or 283 bp for the L and S alleles, respectively.



5HTTLPR MspI restriction digest. The figure above is reproduced from a run from an ABI PRISM® 3130xl Genetic Analyzer. The amplicons are labeled with size in base pairs. The figure above shows all possible restriction fragments, and their source. The red peaks are size standards (Genescan ROX 500).

To summarize, L_G alleles yield 152 bp fragments, L_A alleles yield 326 bp fragments and S alleles yield 283 bp fragments when incubated with MspI. For the following genotypes, the results for the PCR reaction, followed by MspI digest would be:

	PCR	**MspI**
L_A/L_A	419/419	326/326
L_G/L_G	419/419	152/152
L_G/L_A	419/419	152/326
S/S	376/376	283/283
S/L_A	376/419	283/326
S/L_G	376/419	283/152

You may notice that there is a third MspI site 30 bp from the end of the amplicon. This never shows up. Since the enzyme cleaves all of the sites equally well, only the smallest fragment with the 5' fluorescent label is visualized. If you were to run these cut products on an agorose gel, all could be visualized with ethidium bromide or other dye.

To analyze this SNP, the PCR products from above are used. After determining the genotype of the samples from above (e.g., LL, LS or SS), the PCR plate is prepared for MspI (#R106L, NEB, Ipswitch, MA) restriction digest

Sample Preparation for MspI Digest

95 °C 10 min
65 °C 30 min
4 °C hold

MspI Restriction Digest Master Mix for 10 µL reactions

(8 µL Master mix + 2 µL PCR product)

Component	1 Tube vol (µL)	100 Tubes vol (µL)	Concentration of component in: Stock	Master Mix	Reaction
Water	6.8	680			
NEB buffer 2	1.0	100	10 x	0.125 x	1 x
MspI	0.2	20	20 units/µL	4 units	4 units
Total volume (µL)	8.0	800			

MspI Digest Protocol

8 µL of master mix + 2µL 5HTTLPR PCR product

37 °C 3 hours
65 °C 20 min
4 °C hold

MspI Digest Electrophoresis

Size standard mixture for 100 samples:

500 µL water
500 µL Hi-Di formamide
25 µL Genescan 500 Rox

Add 1 µL digest product to 9 µL size standard mix

Samples are analyzed on an ABI PRISM® 3130xl Genetic Analyzer using standard company protocols without modification

Citation: When reporting results for this locus, please cite *Anchordoquy et al, 2003* as the analytical method used for genotyping.

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