Chapter 5

Contribution of Regulatory Element within Repetitive Sequences in Gene Expression: Comparative Functional Analysis between Mouse and Human
5.1. INTRODUCTION

In the previous chapters we have explored contribution of repetitive sequences to promoter divergence and its possible functional consequences. This was primarily carried out to understand its contribution to phenotypic divergence between chimpanzee and human at the level of regulation of gene expression. We identified a few gene regulatory motifs (GRMs) which were significantly diverged (gained or depleted) specifically between human and chimpanzee and were contributed by repetitive sequences. The gains of GRM were also in accordance with its role in transcription of the proximal gene. Processes such as regulation of cell-death had higher number of GRMs which can bind transcription factors known to be involved in apoptosis. This association of repetitive elements to specific functional classes strengthens the previous reports about their non-random association to genomic functions (Grover D et al., 2003; Tsirigos A and Rigoutsos I, 2009). Since our computational study of GRM-divergence was based upon position specific differences in the distribution pattern over 5kb of the orthologous promoter sequences contributed by repetitive sequences between human, chimpanzee and macaque; we tried to functionally validate if these differences could actually contribute to expression differences in downstream genes and if yes then to what extent.

Human and mouse have diverged from their common ancestors ~100mya [a public knowledge-base of divergence times among organisms, Paleontological evidence to date the tree of life]. Mouse has B1 and B2 repeats which are analogous to Alu-repeat from SINE family present in primates. Alu-repeats have been shown to harbour various hormone-receptor elements, vitamin-D receptor elements, retinoic acid receptor elements (RARE), pol-III binding sites as well as many other transcription factor binding sites (Babich V et al., 1999; Gumbart AF et al., 2009; Oei SL et al., 2004). B1 and B2-SINE repeats lack these characteristics. Since several studies have identified presence of RARE in the vicinity of Alu repeats (Laperriere D et al., 2007; Vansant G and Reynolds WF, 1995), we have selected to verify functionality of RARE which were present in Alu repeat at promoter sequences. RAR-elements could regulate expression of downstream genes upon binding with retinoic acid receptors that are activated by the binding of retinoic acid. As the retinoic acid mediated gene regulation involves fewer steps, this system offers an excellent probe to alter the genome hence retinoic acid could act as a molecular handle. We hypothesized that these repeats could
have acquired a functional role during evolution and would be reflected at the expression level. We have selected melanocyte cell-lines which are neuronal in origin and similar genes are conserved between humans and mouse, while some functional differences in pigmentation do exist. Independently retinoic acid has been demonstrated to be important for skin functions. Hence we decided to probe the role of Alu repeats in the RAR mediated gene regulation in melanocyte cell lineage.

5.2. OBJECTIVES

- To identify genes that are up-regulated in both human and mouse after retinoic acid treatment
- To measure RARE distribution in SINE-repeat of promoter from both human and mouse up-regulated genes by retinoic acid
- Selection of a promoter region for functional validation of RARE derived from Alu-repeats
- To measure contribution of individual RARE in regulation of gene expression

5.3. MATERIALS AND METHODS

5.3.1. Identification of differentially expressed genes in mouse melanocyte and human melanocyte primary cell-lines upon treatment with retinoic acid

Human melanocyte primary cells and mouse melanocyte cell-lines have been treated with different concentrations (viz. 10μM, 20μM, 30μM, 40μM and 50μM) of retinoic acid for incubation time of 24 hours. Cells were then harvested and total RNA was extracted using TRIZOL reagent. An Illumina TotalPrep RNA Amplification Kit was used to prepare the samples for hybridization on Illumina arrays. Briefly, first-strand cDNA was synthesized from 500 ng of total RNA, followed by simultaneous second-strand cDNA synthesis and degradation of residual RNA with DNA polymerase and RNase H, respectively. In vitro transcription (IVT) of the purified cDNA was carried out by T7 RNA polymerase using biotinylated primers. The concentration of the purified cRNA solution was estimated using NanoDrop and then hybridized to WG-6 v2.0 and WG-8 v2.0 for Human and Mouse Expression BeadChips, respectively (Illumina, San Diego, CA, USA) following the manufacturer’s instructions. Experiments were carried out using three biological replicates for each treated and
untreated condition. The expression data have been analysed using the Illumina BeadStudio software to identify differentially regulated genes upon treatment with retinoic acid at different concentrations. Optimal concentration of retinoic acid having maximal number of genes having differential regulation has been identified. We have finally selected 158 genes with up-regulation genes upon treatment with retinoic acid at 30μM concentration incubated for 24 hours for further analysis.

5.3.2. Identification of orthologous promoters between mouse-human and putative retinoic acid response elements (RARE)

Since human and mice are very distantly related to each other, the promoter sequences vary a lot. In this case we have used GALAXY (http://galaxy.psu.edu/) tool to fetch the corresponding orthologous promoter for 158 genes identified up-regulated from microarray data analysis. Promoter co-ordinates for human genes have been obtained from UCSC database in the BED file format. This BED file has been given as input to GALAXY-tool to fetch the corresponding mouse region which would be an ortholog for the human promoter. The obtained aligned sequence data have been parsed rigorously to get well mapped orthologs between human and mouse. In-house computational program “PROMO” developed previously in our lab has been used to identify putative RARE motifs on the orthologous promoter sequences of human and mouse. Putative RARE sites having significant scores (>0.9) have been considered for further analysis.

5.3.3. Identification of orthologous promoters that are gained in human due to the differential placement of RARE sites within Alu-repeats

Coordinates of repetitive sequences have been determined through RepeatMasker tool from Repbase (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). Previously identified RARE-sites from 158 orthologous promoters in human and mice were mapped with SINE (Alu in human and B1/B2 in mice) repeats [Table 5.1]. Total number of sites present within SINEs was calculated. Based upon the RARE distribution analysis inside repeats, 12 promoters of genes have been selected which have approximately similar number of RARE sites in human and mouse ortholog but RARE site contributed by SINEs in human have been increased [Table 5.2]. The promoter of KIFIB gene was selected for functional validation of RARE present in Alu-repeat. Figure 5.1 shows distribution of RARE-site at promoter sequence of KIFIB in human and mouse.
Table 5.1: 158 set of genes found up-regulated and for which orthologous promoters could be fetched in both human and mouse upon treatment with 30μM concentration of retinoic acid. The promoters of these genes were studied for RARE-sites present at promoter in human and mouse.

| Gene  | Acvr1 | AdsI | Afg3l2 | Agps | Ak2 | Appbp2 | Ar16ip5 | Arpc1a | Asccl | Atp2a2 | Atg5a1 | Atp6v1b2 | Bace2 | Blcap | Blmb | Bzw2 | Ccnd1 | Ccf5 | Cel5 | Celn2 | Chd1 | Chic2 | Cires2 | Cldn12 | Clns1a | Cml3 | Copa | Cops3 | Cops7a | Cox15 | Crra | Crlk | Crys | Cs | Cse11 | Cstdpl | Dasb2 | Dectn2 | Ddb48 | Dhrs1 | Dhrs7 |
|-------|-------|------|--------|------|-----|--------|---------|-------|-------|--------|--------|-----------|-------|-------|------|------|-------|------|------|-------|------|------|-------|-------|-------|------|------|-------|------|------|-------|-------|------|-------|------|------|-------|-------|------|
|       | Ebhd4 | Elp3 | Fzd6   | Nfia  | Pchp2 | Gjb2   | Pcm1   | Gnb1  | Got1  | Gpiap1 | Gsnf1  | Gfts5a   | Gusb  | Hagh  | Hppl  | Hecf1 | Hibad1 | Hmgb2 | Hmgcl | It30r b| Kdelr2 | Kifib  | Kifip3 | Khi17 | Kpnb1 | Lamp2  | Nfl1   | Lmrn1 | Lta4h | Map1lc3b | Mkm1  | Mkm2  | Mrpl50  | Mrps22 | Mrps31 | Sclk30a5 | Sclk35b3 | Srpcn1 | Smad3 |
|       |       |      |        |       |      |        |        |       |       |        |        |           |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |       |      |        |       |      |        |        |       |       |        |        |           |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

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Table 5.2: Allotment of RARE sites in SINE-repeat present at 5.3 kb promoter proximal region for 158 genes which were up-regulated upon treatment with retinoic acid between human and mouse.

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of common up-regulated genes</td>
<td>158</td>
<td>158</td>
</tr>
<tr>
<td>Total RARE</td>
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<td>746</td>
</tr>
<tr>
<td>RARE in SINE</td>
<td>100</td>
<td>231</td>
</tr>
<tr>
<td>total SINE length</td>
<td>13591</td>
<td>61522</td>
</tr>
<tr>
<td>Percentage of RARE from SINE</td>
<td>14.18</td>
<td>30.97</td>
</tr>
<tr>
<td>Percentage SINE length</td>
<td>1.62</td>
<td>7.35</td>
</tr>
</tbody>
</table>

Figure 5.1: Distribution of SINE repeats and RARE-sites along 5kb promoter region of KIF1B in human (a) and mouse (b). The positive orientation of Alu has been depicted in blue color while repeat in negative orientation has been depicted in brown color. The RARE-sites have been shown as red vertical lines. The first three RARE-site in human cloned region were present inside Alu-repeat and rests of the two RARE-sites were present outside the Alu-repeat. For mouse, all the five RARE-sites were present outside the repetitive sequence.
5.3.4. Validation of KIFIB expression pattern observed from microarray data

To validate the up-regulation event of KIFIB gene analysed from microarray data, real-time PCR based approach was adopted. We identified two alternative transcripts of KIFIB in human and mouse. Probes have been designed to target these transcripts uniquely as well as probes who target both the transcripts. The details of transcripts and the probes positions are given herein [Figure 5.2]. The melanocyte cell-lines for human (WM266.1) and mouse (B16) were treated with 30 μM concentration of retinoic acid. The cells were harvested after 24 hours. Total RNA has been extracted using TRIZOL reagent and 1 μg of this was converted to cDNA using random primers and High Capacity cDNA Reverse Transcription (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as per the manufacturer's instructions.

Figure 5.2: Distribution of SINE repeats, RARE-sites, exonic regions and primer probes for individual transcript isoforms and common transcript regions of KIFIB gene in (a) human and (b) mouse. The C, C1 and C2 primers probe target to both the transcripts whereas U1 and U2 primers target unique transcripts. The 5kb promoter region in colored as green and the 3'UTR is colored as pink.

This cDNA was used for qRT-PCR. qRT-PCRs were performed on a 7900HT ABI platform using 2X SYBR Green master mix (ABI, Life Technologies Corporation, Carlsbad, CA, USA). The relative mRNA expression level analyses were carried out using the 2^(-ΔΔCT) method. The observed data has been analysed to measure the expression differences upon retinoic acid treatment compared to untreated cells.
5.3.5. Constructing a reporter plasmid with ~2.5 kb promoter of KIF1B from human and mouse into firefly luciferase vector system with minimal promoter.

Promoter sequence region have been identified for making the reporter construct having RARE-sites within Alu-repeat in human and a corresponding orthologous sequence from the mouse. The region with interest has been amplified using PCR [Figure 5.3].

Primers for cloning

**Human KIF1B promoter:**

Forward Primer: TTGGTACCGAGGCAAAAGGCTTATGC  KPN I

Reverse Primer: AAGACCTTCACCTACGCAGCAGC  HinD III

**Mouse KIF1B promoter:**

Forward Primer: AAAGTACGGAGGATGCCATCTGTTTGG  KPN I

Reverse Primer: TTGGTACGGTCCTCCCGGAGGC  Nhe I

Figure 5.3: Gel picture with PCR amplified promoter region from mouse and human have been extracted and were cloned in firefly luciferase vector (pGL4.23).
We have selected a region of 2610bp from human KIFIB promoter and 2213bp from mouse KIFIB promoter to clone it into luciferase vector pGL4.23 (luc2/minP) having minimal promoter, were made with Alus harbouring putative RARE sites as well outside Alu as insert. The templates were amplified by PCR from human/mouse genomic DNA followed by gel purification of PCR products using Qiagen columns prior to cloning. The clones were confirmed by sequencing.

5.3.6. Transfection of reporter construct into human and mouse melanocyte cell-lines followed by dual luciferase assay (DLR-assay) and analysis

The reporter constructs for human and mouse KIFIB promoter has been transfected with Cellfectin reagent form Life Technologies as per manufacturer’s protocol into human and mouse melanocyte cell-lines followed by treatment of cells with 30μM retinoic acid. 1 μg of each construct in pGL4.23 was co-transfected with 50 ng of pGL4.75 renilla luciferase (Promega, Madison, USA) as a control. After 24 hours the cells were harvested to get the cell-extract and the measurement of luciferase enzyme has been carried out through dual luciferase assay technique. Retinoic acid which induces the RARE sites through retinoic acid receptors will bind to RARE and promote up-regulation or down-regulation or the reporter gene (firefly luciferase in this reporter construct). A background measurement of renilla luciferase has been used as control to calculate the differences in luciferase activity from reporter constructs between treated and controlled samples. Computationally, in the promoter that drives the renilla luciferase RARE sites could not be identified, and preliminary experiments did not show appreciable change in the activity upon retinoic acid addition. Hence this was chosen as a normalizing control. The measurement of luciferase activity gives indication for the amount of enzyme made and which is the indication of cloned promoter activity. The empty vector has been used as the negative control.

5.3.7. Site directed mutagenesis (SDM) to measure effects of individual RARE sites on basal expression of luciferase enzyme

By using SDM protocol, we mutated the putative RARE-site to disrupt the binding of regulatory protein which will affect its function in terms of variation in luciferase gene...
expression and which could be measured. Clones were subjected to site-directed mutagenesis using a QuikChange II XL kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's protocol to incorporate desired base changes in the predicted RARE sites within Alus. In each of the clones, the four most-conserved bases were mutated. Site-directed mutagenesis clones were confirmed by ABI sequencing. In this way impact of individual RARE-sites has been measured by mutating each and every RARE-site in reporter construct for human KIF1B promoter.

We made 5 different clones with individual RARE mutants and also a combination of mutants (viz. 1-2, 1+2+3, 1+2+3+4, 1+2+3+4+5 and 4-1-5) to assess their combinatorial affect on gene regulation.

**Positions and Forward Primers for SDM on human KIF1B cloned promoter**

The positions of the SDM sites have been shown as red text and the primer sequences are represented as green text. The desired modifications have been introduced to create five mutations at four consecutive sites at the core region of the RARE-site and they are represented as black text within the primer sequences. The first mutation has been names as M-1 and later on as we move towards the TSS, the numbers increase till M-5. First three RARE-sites are present inside Alu-repeats shown in gray color text and the last two RARE-sites are present outside the repeat but inside in CpG Island (not shown).

>human_KIF1B_promoter

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5.4. RESULTS AND DISCUSSION

5.4.1. Identification of genes with up-regulated expression from human melanocyte primary cell-line and mouse melanocyte cell-line treated with retinoic acid

Treatment of human melanocyte primary cell line and mouse melanocyte cell line (B16) by retinoic acid at 5 different concentrations for 24 hours has been followed by gene expression analysis. We found most of the genes were differentially regulated at 30μM concentration of retinoic acid in both human and mouse melanocyte cell-lines. A total of 241 genes were identified to be up-regulated in both the cell-lines among which ortholog information for 158 genes promoter could be fetched. These genes have been taken for further study to identify putative RARE sites present in 5kb promoter of them.

5.4.2. Distribution of putative RARE sites at orthologous 5kb promoter of up-regulated genes in human and mouse

Identification of putative RARE sites in 5kb promoter sequences has identified their presence in both repetitive (Alu-repeat) and non-repetitive regions. When we compared the total number of RARE sites in promoter between selected 158 human and mouse genes, we observe not much difference in them since mouse promoter had a total of 705 RARE sites while human promoters had 746 RARE sites. When we looked for the percentage of RARE which were contributed by Alu-repeats, we observed that ~14% and ~31% of the RARE sites are contributed by SINE in mouse and human respectively. Also there was a remarkable increase in the total base composition of SINE in the human lineage [Table 5.2].
5.4.3. Orthologous genes with their promoter having equal number of RARE whereas Alu-repeats have gained RARE in human

We have identified 12 genes from the set of 158 genes which have been found up-regulated upon treatment to retinoic acid on the basis that both of them have promoters with approximately equal number of RARE sites. Simultaneously there should be more number of RARE contributed by Alu-repeat in human. These 14 genes along with their RARE sites in SINE (Alu in human and B1/B2 in mice) are tabulated [Table 5.3]. This set of genes represents to us the ones where the functional diversification is brought about by the Alu elements while retaining the overall regulatability of the gene by retinoic acid.

Table 5.3: 12 selected genes wherein about similar number of RARE were present in promoter between human and mouse. Also there was a gain of RARE within Alu-repeat (SINE) from human.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total RARE in mouse</th>
<th>RARE in SINE in mouse</th>
<th>Total RARE in human</th>
<th>RARE in SINE in human</th>
<th>Total RARE gained in human</th>
<th>Total RARE gain in human inside SINE</th>
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<td>7</td>
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</table>

5.4.4. Validating results from microarray data about KIFIB gene-expression for treatment with retinoic acid

qRT-PCR analysis for measurement of expression of individual transcripts and the whole gene for KIFIB has been carried out in human and mouse melanocyte cell-lines. The qRT-PCR analysis has confirmed the up-regulation of KIFIB gene expression
upon treatment with 30μM concentration of retinoic acid for 24 hours [Figure 5.4]. Both the transcripts of KIFIB gene in human and mouse have been found up-regulated but the fold change was bigger in mouse as compared to the human KIFIB transcripts.

Figure 5.4: KIFIB transcripts level upon retinoic acid treatment compared with and untreated condition in human and mouse melanocyte cell-lines. Kif_U1 and Kif_U2 represent primer probes targeting unique transcript isoforms of KIFIB gene whereas Kif_C represents primer probe targeting both the transcripts. Human KIFIB gene is showing less induction upon treatment with retinoic acid whereas mouse KIFIB gene expression is more than 2 folds.

5.4.5. Functional validation of putative RARE harboured by Alu-repeat

Reporter constructs with ~2.5kb promoter harbouring several RARE sites in promoter sequence of KIFIB gene from human and mouse have been transfected in melanocyte cell lines to identify the functional activity of cloned promoters. Results analysed from dual luciferase assay represents up-regulation of firefly luciferase enzyme from both human and mouse promoter reporter constructs which suggests the KIFIB promoter’s positive role in regulation [Figure 5.5]. We next look to identify the individual site contribution to this regulation. For this we adopted site directed mutagenesis procedure to disrupt each individual RARE site by mutating the 4 bases in the core base sequences. We observed that not all the RARE sites have similar affect to the luciferase gene. First and fifth RARE sites were shown to have more drastic affect as compared to the rest ones [Figure 5.6].
Figure 5.5: Figure represents induction of promoter activity of human and mouse reporter constructs upon treatment with retinoic acid in human melanocyte cell-line. The increase in promoter activity in response to retinoic acid in both human and mouse KIF1B gene validates the functionality of these sites.

Figure 5.6: Basal expression of promoter activity from reporter constructs of wild type and mutant reporter constructs. (a) All the mutants show higher promoter activity upon treatment with retinoic acid. (b) A detailed view for the figure-(a), although there is up-regulation of promoter activity upon treatment with retinoic acid, there is change (either up or down) in the basal expression value. Mutations at 1st RARE site and 5th RARE site have remarkable decrease in the basal promoter activity. Since 1st RARE was present in Alu-repeat, it suggests Alu-repeat functional involvement in up-regulating the downstream gene-expression.
Since first RARE site was present a distance more than 2kb from the KIF1B transcription start site (TSS) and also inside the repeats, this suggests that Alu-repeat could have a functional impact on the up-regulation of KIF1B gene. Such exaptation of repetitive sequence have been reported previously (Bejerano G et al., 2006; Shankar R et al., 2004).

5.4.6. Additive effect of RARE present at the promoter sequences

The analysis regarding the study of RARE sites without their combinatorial effect on regulation of gene-expression would be incomplete since it is already known that GRMs work in a combined manner to control the downstream genes and manage their interaction through stimulus from environmental signals. We wanted to explore about RARE sites’ combined effect whether they were additive or not. To do this we have made reporter constructs having several combinations of RARE-mutations (viz. 1+2, 1+2+3, 1+2+3+4, 1+2+3+4+5 and 4+5). We transfected these constructs in human melanocyte cell-lines and carried out dual luciferase assays [Figure 5.7].

Figure 5.7: Basal expression of promoter activity from reporter constructs having a combination of RARE-site mutation from human KIF1B promoter. The results show that there is generally a gradual decrease in basal expression for un-treated and retinoic acid treated when transfected into human melanocyte cell-lines. Another major observation is that expression of luciferase gene having reporter construct with mutation 1 and 2 have higher basal expression when compared to their individual mutation constructs from previous figure.
We observed that there was a recognizable pattern of decreasing basal expression with systematic abolishment of RARE sites by the mutations. This suggests that GRMs could act independently and their effect could add-up to bring a drastic effect on the regulation of gene-expression. Another observation looked to be contrasting with previous one when we look at the basal expression of luciferase gene with reporter constructs having 1\textsuperscript{st} and 2\textsuperscript{nd} RARE mutations within them having high basal expression when compared with the wild-type as well as construct with only mutation at 1\textsuperscript{st} RARE site. We have looked for the DNA-sequence characteristics of locations for these RARE sites and found that RARE sites at position 4\textsuperscript{th} and 5\textsuperscript{th} were present at CpG sites with high methylation properties. This could be quite possible that RARE sites present at distant locations work as enhancers and interact with sites present in 4\textsuperscript{th} and 5\textsuperscript{th} positions because 4\textsuperscript{th} and 5\textsuperscript{th} RARE mutations alone have shown drastic decrease in basal expression [Figure 5.6]. The results of the analysis indicate a more complex association of GRMs where repeats could also be involved in active manner to control the regulation of gene expression and a deeper understanding need to be developed to delineate the functional role.

5.5. CONCLUSION

Results of the present work suggest a central role of repeats in regulation of its neighbouring genes. However repeats present at different locus may not have similar functional impact upon regulation, they may have combined effect. Although in some cases these combined effects could be different with their individual impact. Our analysis supports the hypothesis regarding functional aspect of repetitive elements thus provide an essential contribution towards functional variation across genomes by bringing simple resource of variation in promoter sequences.
5.6. REAGENTS AND PROTOCOLS

Media

**LB-broth**: (for one litre)

- Tryptone: 10 gm
- Yeast extract: 10 gm
- NaCl: 5 gm
- Dissolved in 1 litre of Milli-Q water (ph 7.0)
- Autoclaved and stored at room temperature.

**LB-agar**: (for 1 litre)

- LB-Broth + Agar (15 gm)
- Autoclave and store at room temperature
- When in need, de-solidify in microwave and pour on Petridish
- If carbenicilline need to be added, add 250 μl of carbenicilline in 250 ml of LB-agar.

Antibiotic stocks

**Carbenicilline**: (more stable at lower pH than ampicilline)

- 50 mg / ml of Milli-Q water.
- Store at -20°C

Solutions for chemical competent cells

**DH5-medium**: 

- Bactotryptone: 20 gm
- Yeast extract: 5 gm
- MgSO₄: 5 gm
- Dissolved in 1 litre Milli-Q water
- pH adjusted to 7.6 with 1M KOH
- Autoclaved in 250 ml portions

**TFl-buffer**:  

- 1M Potassium Acetate: 3 ml (0.29 gm)
- 1M KCl: 10 ml (0.75 gm)
Chapter 5

Buffers for the isolation of DNA

Resuspension Buffer (P1): (for 1 litre)

- Tris-Cl 50 mM
- EDTA 10 mM
- RNAse 10 mg/ml
- pH to 8.0 with HCl
- 6.06 gm Tris has been dissolved in 800 ml of Milli-Q water
- 3.75 gm EDTA.2H2O dissolved in it.
- pH adjusted 8.0 with HCl
- Volume made up to 1 litre with Milli-Q water
- Autoclaved and cooled
- 10 ml RNAse-A (10 mg/ml stock) added and stored at 4°C.
Lysis buffer (P2): (for 1 litre)

- NaOH 200 mM
- 1% SDS
- Dissolved 8.0 gm NaOH pellets in 950 ml autoclaved Milli-Q water
- Add 50 ml 20% SDS solution (made in autoclaved Milli-Q water)
- Filter, sterilize and stored at room temperature.

Neutralizing buffer (P3): (for 1 litre)

- 3M Potassium Acetate (pH 5.5)
- Dissolved 294.5 gm Potassium Acetate in 500 ml Milli-Q water
- pH adjusted 5.5 with Glacial Acetic acid (~110 ml)
- Volume made up to 1 litre with Milli-Q water
- Autoclaved and stored at room temperature.

RNAse-A (10 mg/ml): 

- Add 1 ml of 0.5M Tris pH 7.5 and 1.5 ml 0.5M NaCl to 25 ml Milli-Q water
- pH adjusted to 8.0 with HCl
- Volume made up to 50 ml
- Autoclaved and cooled
- Dissolved 0.02 gm RNAse-A in 2 ml of [10 mM Tris (pH 7.5) – 15 mM NaCl buffer] prepared above
- Aliquot into 2 micro-centrifuge tubes (1 ml each) and heated at 100°C for exactly 15 minutes
- Cooled at room temperature and stored at -20°C.

Buffers for RNAse-A preparation

0.5 M Tris (pH 8.0):

- Tris 6.055 gm
- Dissolved in 80 ml of Milli-Q water
- pH adjusted to 8.0 with 0.1M HCl (~20 ml)
- Volume made up to 100 ml with Milli-Q water
- Autoclaved and stored at room temperature.
100 mM EDTA:
> EDTA 3.722 gm
> Dissolved in 80 ml of Milli-Q water
> pH has been adjusted to 8.0 with 1M NaOH (~12 ml)
> Volume made up to 100 ml with Milli-Q water
> Autoclaved and stored at room temperature.

0.5 M NaCl:
> NaCl 2.922 gm
> Dissolved in 100 ml Milli-Q water
> Filter, sterilized and stored at room temperature.

Solutions for blue white selection of recombinants

X-gal:
> X-gal 20 mg / 0.020 g
> Dissolved in 1 ml di-methyle-formamide (DMFA)
> Tube containing the solution was wrapped in aluminum foil to prevent damage by light and stored at -20°C.
> Its final concentration in LB-agar should be 80μg/ml.
> So one ml of the solution has to be mixed in 250 ml of LB-agar.

IPTG:
> IPTG 0.2 g
> Dissolved in one ml of Milli-Q water
> Filter, sterilize and store at -20°C.

Digestion & Ligation

Restriction enzyme digestion:
1) MQ 42.3μl
2) Plasmid DNA 2.0μl
3) Buffer 5.0μl
4) 100x BSA 0.5μl
5) Enzyme 0.2µl
6) Total volume 50µl
7) Take 100µl of total reaction in 5 tubes of 20µl each and incubate 5hrs to overnight at 37°C.

CIP treatment:
1) Calf Intestinal Phosphatase (CIP) treatment is required to prevent the self ligation of the bluntly digested plasmid as it removes the -po4 from the 5' end of the DNA.
2) Each of the 20µl of digested mixture has to be treated with 1µl of CIP enzyme and incubate for one hour at 37°C.
3) Purify the DNA using the PCR product purification protocol and store the DNA at -20°C.

PNK treatment of insert:
1) Polynucleotide Kinase (PNK) enzyme treatment of the insert DNA is necessary to introduce the -po4 bond at the 5' end of DNA so that it could bind to the plasmid easily.
2) DNA insert 30µl
3) PNK enzyme 1µl
4) Nuclease free water 15µl
5) T4-DNA ligase buffer 5µl
6) Total volume 50µl
7) Incubate at 37°C for one hour.
8) Purify the DNA using the PCR product purification protocol and store the DNA at -20°C.

Ligation step:
1) For 10µl of reaction
2) Insert DNA 7µl
3) Plasmid DNA 2µl
4) T4-DNA ligase buffer 1µl
5) T4-DNA ligase enzyme 1µl
Protocols

Making competent cells:

1) Streak the agar plate with XL1-blue cells from the stored vial and let it grow overnight at 37°C to pick up colony.
2) Next day morning check for the colonies grown and transfer the plate at 4°C to preserve/store.
3) On evening make the inoculum of XL1-blue cells in LB media by picking up the single colony from the plate and transfer it to the DH5α-media in a 50 ml falcon tube.
4) Then incubate it overnight at 37°C.
5) Next day morning take 500μl of inoculum which has been taken from overnight grown culture, has been added to 50 ml of DH5α medium in a conical flask. Incubated at 37°C on shaker for 2 hrs approximately, till its OD becomes 0.5 units at A600 nm.
6) Store on ice for 10 minutes.
7) Then this culture has been separated into two chilled falcon tubes of 50ml (25 ml each) and then centrifuged at 3000 rpm for 15 minutes at 4°C. Keep in mind that all the equipment should be chilled.
   (Note: the cells should not be in contact to higher temperature than 4°C at any step)
8) Now discard the supernatant carefully inside the hood and add (10ml) of TFI buffer to each tube for washing the cells.
9) Centrifuge this to 3000 rpm for 15 minutes at 4°C.
10) Resuspend the cells using TF2 buffer (1ml) and make the single tube by adding the two tubes.
11) Aliquot the cells into 1.5ml eppendorf tubes (100μl each) which has been crosslinked and chilled before aliquoting the competent cells.
12) Now competent cells have been snap frozen in liquid nitrogen and then stored at -80°C.
To check the efficiency of prepared competent cells:

1) The efficiency of competent cells can be checked by plating 100 µl of competent cells which has been transformed with 1ng, 10ng and 100ng of pDNA of vector (PGL 4.75 in my case) and control (with no DNA).

2) All the transformation procedure has been applied and plating of the transformed cells has been done on carbenicilline agar plates.

3) The colonies have been incubated at 37°C for overnight.

4) Counting of the colonies has been done to find out the efficiency of competent cells.

5) For good efficiency there should be $10 \times 10^6$ colonies/µg of plasmid DNA whose length is ~3000 bp.

Vector transformation:

1) Take XL1-blue competent cells vial of E. Coli from -80°C freezer and thaw on ice (~10 - 15 minutes)

2) Take 100µl of competent cells (for ligated DNA product) / 20µl of competent cells (if just for amplification of cells) in a chilled eppendorf

3) Add 0.5µl of DNA (if ligated product) / 0.5µl to 1.0µl of DNA (for amplification of cells) to competent cells.

4) Incubate on ice for 30 minutes.

5) Give heat-shock at 45°C for exact 90 seconds.

6) Snap chill the tubes for 5 minutes on ice.

7) Add 500ul of LB medium and incubate the tubes at 37°C for 45 - 60 minutes for cells revival.

8) Plate the solution on antibiotic plate or as desired.

9) Incubate the plate at 37°C for overnight to grow the colonies.

10) Keep the plates pack with paraffin and store at 4°C.

Isolation of plasmid DNA:

1) Take 1.5ml overnight grown culture in micro-centrifuge tube.

2) Centrifuge at 4500 rpm for 5 minutes (pellet could be stored at -20°C).

3) Add 250µl buffer P1 with RNase-A.

4) Vortex gently and dissolve the pellet completely (don’t over-mix).
5) Add 350\,\mu l of buffer P2 and shake it by inverting the tube gently.
6) Add 350\,\mu l of buffer P3 to precipitate the proteins on walls by rotating the tube in circular motion.
7) Centrifuge the tubes at 12000 \,g for 15 minutes.
8) Transfer the supernatant to a new tube.
9) Add 700\,\mu l iso-propanol to each tube.
10) Incubate 10 minutes at room temperature.
11) Spin at 13000 \,rpm for 15 minutes.
12) Discard the supernatant.
13) Wash the pellet with one ml of 70\% ethanol and spin 5 minutes at 13000 \,rpm.
14) Dry the pellet after removing the supernatant.
15) Resuspend in 30\,\mu l of autoclaved Milli-Q or nuclease free water.
16) Store this DNA at -20\,\degree C.

Transfection in cell-line:

1) The clones which have been made for the promoter of KIF1B, has to be transfected in a suitable cell-line to get expressed.
2) I have used the dual luciferase assay which is to measure the firefly and renilla Lucifer enzymes one after another, so in this system there is a simultaneous transfection of the cells with firefly and renilla vectors. The renilla vector has a very strong constitutive promoter so as to express a sufficient amount of protein all the time. This renilla vector expression is generally taken as the internal control to balance the measurements of the firefly luciferase enzyme.
3) In our case the experimental DNA has been cloned to the firefly luciferase vector at promoter of the luciferase gene which has a very weak promoter. This vector is suitable for the analysis of regulatory effect of the DNA which could modulate the expression of the downstream luciferase gene.
4) In dual luciferase assay, there is co-transfection of the cloned vector (in pGL4.23) and renilla (pGL4.75) in 19:1 ratio to each well of 6-well plate.
5) Total DNA amount for the transfection should be 1000ng (950:50ng for pGL4.23 and pGL4.75).
6) For each well of the 6-well plate, 4µl of the cellfectin (transfecting reagent) have been used.

7) DMEM-F12-SFM (Serum Free Media) has been used to mix the cellfectin and the experimental and control vector DNA.

8) First, consider that all wells of the 6-well plate has to be transfected, so for the 6 wells, 600µl media has been taken in the 1.5 ml eppendorf tube and 24µl of cellfectin have been mixed and vortex it. Same time prepare 6 eppendorf tubes separately to with 100µl media each and add them with 950ng of experimental clone DNA vector and 50ng of renilla vector DNA. Now add the 100-100µl of cellfectin media to each of the DNA vector eppendorfs. Now gently vortex the mix and incubate for 15-20 minutes.

9) During this incubation period, wash the cells with DMEM-F12-SFM twice (confluency of cell should be 30-40%).

10) After the incubation, add the mix (200µl) to the empty wells and then add 800µl of the DMEM-F12-SFM media.

11) Incubate the plate at 37°C for 4-8 hours and then replace the media.

12) Next day, trypsinized the cells from each well separately and each has been divided in to two or four wells of the 24 well-plate according to the need.

13) After 18-24 hrs, cells have been treated with 30µM concentration of all-trans-retinoic acid and incubated for 24hrs. at 37°C.

14) Next day, cells have been harvested with passive lysis buffer (PLB) and taken for the measurement of luciferase activity by dual luciferase assay.
5.7. REFERENCES


Tsirigos A, Rigoutsos I (2009). Alu and bl repeats have been selectively retained in the upstream and intronic regions of genes of specific functional classes. *PLoS.Comput.Biol.* 5:e1000610