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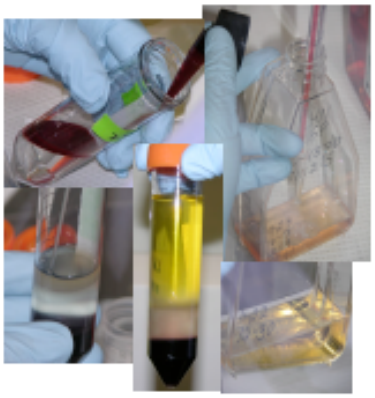
FEBRUARY 8, 2006 VOLUME 2, NUMBER 1

...To Meet Your Research Needs In Diabetes And Diabetes Complications...

DERC HUMAN GENETICS CORE



With the rapid advances that are occurring in the HapMap, high throughput genotyping, and statistical analysis methodology, there are finally beginning to be some meaningful advances in the identification of genes contributing to complex disorders such as diabetes.

The goal of the Human Genetics Core is to offer expertise to DERC investigators conducting studies into the genetics of diabetes, its complications and related endocrine disorders. The Core provides access to both the expertise and facilities necessary for such genetic research in human populations.

Core Services:

- Establishment and maintenance of EBV-transformed lymphoblastoid cell lines and generation of nonviable cell pellets for DNA/RNA isolation
- Access to anonymized lymphoblastoid cell lines from subjects well characterized for diabetes and/or insulin sensitivity for such purposes as searching for mutations in specific candidate genes *(in development)*
- Genotyping services including genome scans, microsatellite and SNP testing of candidate genes
- Assistance with genetic study design
- Genetic statistical analysis assistance
- Training for DERC investigators and their staffs to perform many of these procedures themselves, with ongoing consultative support from Core staff

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THE LYMPHOBLASTOID CELL LINE FACILITY

The Lymphoblastoid Cell Line Facility was established at Cedars-Sinai Medical Center in 1986, as part of the Common Disease Genetics Program. The justification for this facility was a growing appreciation that efforts to locate and clone the genes implicated in these non-Mendelian, oligogenic conditions would require both detailed physiologic evaluation of families in which these diseases clustered and extensive molecular genetic laboratory investigation. Because of the substantial time and financial efforts needed to identify, recruit and phenotype large numbers of subjects, having reliable access to large quantities of DNA was vital to maximize the utility of such clinical investigations. Ebstein-Barr Virus transformation of B lymphocytes results in immortalization of lymphoblastoid cell lines that can then be maintained indefinitely. Beginning in 1999, when a GCRC Genotyping/Phenotyping Core was funded, it became possible to expand the Cell Line Facility and to make LB cell line establishment a service available to all GCRC-authorized investigators at Cedars-Sinai and also at Harbor-UCLA Medical Center, our parent GCRC. When the DERC was funded, it became possible to offer this service to DERC investigators at all four campuses – Cedars-Sinai, UCLA, UC San Diego, and Salk Institute.

LYMPHOBLASTOID CELL LINE METHODOLOGY

Permanent lymphoblastoid cell lines are established by Epstein-Barr virus transformation of lymphocytes isolated from peripheral whole blood according to the method of Anderson and Gusella (1) and Miller and Lipman (2) and adapted specifically for our laboratory (3). The lymphoblastoid cell lines are monitored for contamination (bacterial, fungal, and mycoplasmal). Cell lines are carefully frozen to ensure maximum viability upon thawing. At the time that cell lines are ready for freezing, cell pellets containing approximately 20 x 106 cells are aliquoted for DNA and frozen non-viably, but this number can be modified depending upon the needs of the DERC investigator. If immediate molecular

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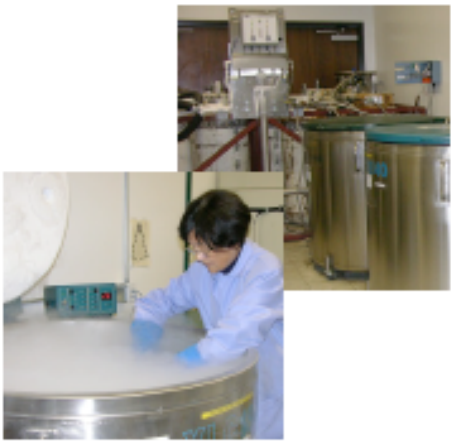
studies are planned, a cell pellet can be given to the investigator or to the Genotyping Laboratory without being frozen.

Our initial successful transformation rate is > 95%. If any sample is unsuccessful, we contact the study team and request that another sample be obtained from the subject, if possible. When getting a second blood sample from a study participant for transformation, we recommend that several purple top, EDTA tubes also be drawn. This allows backup buffy coat to be stored, in the rare event that the second sample also fails to transform. We generally do not recommend trying to establish a cell line more than twice.

EXISTING LYMPHOBLASTOID CELL LINES

Previously transformed and frozen lymphoblastoid cell lines can be thawed and regrown if a DERC investigator has utilized all of the non-viable cell pellets that were initially aliquoted, or if the investigator requires viable cells in culture (as for example, may be needed for RNA isolation for expression studies to be performed by the Transcriptional Genomics Core). T25 flasks, containing approximately 25 x 106 are typically what will be provided, but larger quantities may be obtained if needed for specific studies.



CREATION OF AN ANONYMIZED LYMPHOBLASTOID CELL PANEL

Over the years that the Lymphoblastoid Cell Line Facility has been in existence, we have had many requests from investigators to be given access to existing lymphoblastoid cell lines to use as for a variety of indications. One common request has been for cells from a particular ethnic group to either be able to determine allele frequencies for a newly identified genetic polymorphism or to test if a newly identified variant occurs in the general population (and thus is less likely to be a disease-causing mutation) or not (in which case it may indeed be a disease-associated mutation). Another request has been for repetitive access to a particular cell line to be used as a control in various bench lab experiments. Lastly, some investigators have requested cells from individuals with a particular phenotype (i.e. diabetic subjects vs. non-diabetic subjects) to look for differential gene expression. Due to issues of confidentiality, anonymity and consent, the lab had been unable to provide this service. After working closely with the IRB to develop a confidential procedure, have now begun establishing anonymous panels of cell lines, for which limited phenotypic information remains accessible. To date, 85 cell lines have been anonymized. DERC investigators can now access these cell lines for their experiments, without concerns of potential breach of confidentiality.

The primary panel of cell lines is being established using cell lines from subjects who were studied as controls in various studies. Thus, while these subjects are not guaranteed to have been healthy, they were not recruited because they had a specific disease. The information retained with these samples includes: age at sample acquisition; approximate year of birth (i.e. 1970-75); gender; ethnicity.

Table : Proposed Make-up of Anonymized Lymphoblastoid Cell Line Panels

		Proposed Number of Anonymized Cell Lines per Group		
Ethnicity	**Sex**	**Phenotype**		
		Diabetes	No Diabetes but Insulin Resistance	No Diabetes and Insulin Sensitive
Hispanic	M	20	20	20
	F	20	20	20
Non-Hispanic Caucasian	M	20	20	20
	F	20	20	20
African American	M	20	20	20
	F	20	20	20
Total		***120***	***120***	***120***

In addition, we are in the process of developing three panels of anonymized cell lines, based on diabetes or insulin sensitivity, as detailed in the Table below. Information being retained following anonymization will include: age (at sample acquisition), approximate year of birth (i.e. 1970-75), gender, ethnicity, presence or absence of Type 2 diabetes, and for non-diabetic subjects, whether the

subject was insulin resistant or insulin sensitive. When feasible, the non-diabetic cell lines will be selected from subjects who have undergone formal measurement of insulin resistance by hyperinsulinemic euglycemic clamp (4). If insufficient numbers of subjects with clamp measurements are available in a given ethnic or gender category, then insulin resistance/sensitivity will be defined by homeostasis model assessment (HOMA) (5).
DERC investigators will be able to request both non-viable cell pellets for DNA extraction and viable cells in culture. Because each specimen will have a panel ID number, an investigator who wishes to receive additional cells from a particular panel lymphoblastoid cell line will be able to do so. We anticipate that the initial groups of diabetic, insulin sensitive and insulin resistant cell lines will be available beginning in early summer, 2006.

GENOTYPING LABORATORY SERVICES

A variety of genotyping technologies are available to DERC investigators, for use in either linkage or association-based study designs. Investigators have the option of using microsatellites or snps (single nucleotide polymorphisms).

GENOME SCANS

A primary advantage of whole genome mapping is that it can use PCR markers that can be typed by semi-automated, high throughput methods. Chromosome specific microsatellite sets of PCR markers for fluorescence-based genotyping have been developed. PCR marker sets are available from Applied Biosystems (ABI PRISM®). These sets consist of microsatellite markers with compatible, non-overlapping size ranges, that can be run together in a single lane on a gel. This allows one to analyze 5 to 7 different microsatellite loci in a single lane. By using three different fluorescent dyes, then 15 to 18 microsatellite loci can be analyzed per lane of a sequencing gel. The Genescan software available from Applied Biosystems (ABI) can be used to measure allele sizes of loci run on the ABI fluorescent DNA sequencing machine by including size standards in each lane that are labeled with a fourth fluorescent dye that is not used for PCR. The Genotyper software can then determine the Mendelian inheritance of the alleles by referring to the family structure. The biggest advantage of using the ABI DNA sequencer to analyze microsatellites is that it determines the exact size of each allele so that the data can be used to test for the specific effects of individual alleles. This type of absolute allele assignment has been very slow and difficult with 32P-labelled microsatellite markers. The next most important reason for using the semi-automated approach to genotyping is that the number of genotypes determined per laboratory personnel is higher than for manual genotyping using 32P-labelled PCR products.
The ABI system contains software that first processes the gel electrophoresis data, calculates the molecular weight of the PCR products with respect to internal lane standards, and converts these molecular weights to sizes of alleles. Each allele for each subject in each gel is visually examined using this software. The use of internal lane standards gives consistency in allele calling as the Cedars-Sinai GCRC Genotyping laboratory has observed PCR products to migrate within 0.2 bp when run on gels 6 months apart. The laboratory has written subroutines using the data handling software FileMarkerPro and FoxPro to process these data further and prepare files for analysis.

MICROSATELLITE MARKERS

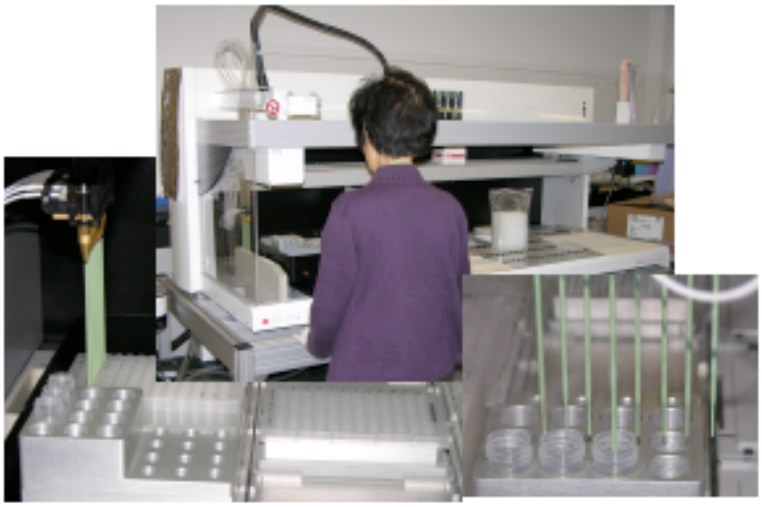
Genotyping of candidate genes and/or fine mapping using microsatellites utilizes the same basic molecular methodologies as for genome scans but with markers that are not of a standard panel. Investigators are requested to provide detailed documentation for the microsatellites they wish to use when these have been pre-selected. If a DERC investigator is interested in testing a particular gene or locus, but does not have specific markers selected, Dr. Taylor and his staff will assist with bioinformatic searches to identify potential markers near or within the gene of interest.

Once the markers have been selected, we typically redesign the primer and PCR conditions for the selected marker

using Genbank information and primer design software (Oligo 6.0®) to optimize the possible PCR conditions. This step greatly minimizes the rate of missed genotypes on the first pass and thus decreases the workload.

SNP GENOTYPING

SNP genotyping is performed using TaqMan MGB technology in a 384-well format (ABI 7900; 6). There have been several recent improvements to this technology: 1) higher throughput 384-well format, 2) replacement of TET dye with VIC dye makes the fluorescence of the two alleles more even, 3) use of a minor groove binder (MGB) allows the use of shorter probes and thus increases the allele discrimination (7), 4) design software that minimizes the trouble-shooting of each individual reaction (8), 5) an improved plate cap and optical system that minimizes the failure of individual reactions because tubes were not capped properly, and 6) reactions may be performed in any PCR machine and "read" by the ABI 7900, thus increasing throughput. To maximize efficiency, we use a Tecan Genesis robotic workstation to set up the PCR reactions and feed into the ABI 7900. Further dye developments promise to increase throughput in the future (9). In our hands, 80% of SNP reactions can be designed using the software and the reaction set up without trouble-shooting, 10% require trouble-shooting, and the remaining 10% require an alternative technology.



If the TaqMan MGB design is not possible and a given SNP must be genotyped, we will use: 1) restriction enzyme digests, with natural sites (e.g. 10, 11) or with engineered sites (e.g. 12), either with 100-well agarose gels or with laser-activated dyes (ABI 377); or, 2) allele-specific oligonucleotide probes hybridized to PCR products dotted on nylon filters in 96-well format followed by a tetramethylammonium (TMAC) wash (Amersham Hybond N+ protocol, 13, 14). The TMAC washing procedure gives a 4ºC difference in the wash temperature between matched probe and mismatched probe for very reliable allele discrimination. Dotting permits multiplex SNPs for higher throughput (15).
Data are handled using FileMakerPro and Access database software will be transmitted to the analytical team using the Cedars-Sinai computer network infrastructure, or will be transmitted electronically directly to the DERC investigator.

Illumina Oligo Ligation Assay/Micro Bead Technology for High-Throughput Genotyping

The core has recently acquired an Illumina BeadReader station and now has the capacity for very high throughput SNP genotyping with custom designed arrays. This allows SNP genotyping on the scale of 384, 768, or 1536 SNPs in 800 samples (310,000, 610,0000, or 1.2 million genotypes per run) at costs that are rapidly falling. Features of this technology are (16): (1) computerized bioinformatics and assay design, (2) high-throughput robotics, (3) automated oligonucleotide synthesis and bead attachment, (4) oligonucleotide ligation assay (OLA) (17), (5) "zipcode" sequences in order to decode the multiplex OLA reactions (18), (6) zipcode attached to microsphere bead (19), and (7) optical readout of beads by fiber optic array (20). Quality-control features: (1) SNPs are first run through a design algorithm that eliminates assays with known problems in the sequence prior to synthesis of reagents, (2) each assay is repeated a mean of 30 times with a minimum of 6 times for each subject, and (3) assays are rejected (~15-20%) that do not give excellent separation between the alleles when read in the fluorescence detector. For quality control, we include several random duplicate samples (~5%) and each SNP assay is tested for Hardy-Weinberg equilibrium.

BOINFORMATICS CONSULTING

Investigators requiring assistance in identifying microsatellite or SNP markers appropriate for their research activities, or assistance in locating potential candidate genes of interest can consult with Dr. Taylor. Depending on the complexity of the search, Dr. Taylor will either perform the search, or instruct the DERC investigator or research staff on how to access public databases, such as Genbank, MDECODE and the NCBI dbSNP, and how to utilize the various search engines.

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GENETIC EPIDEMIOLOGY SUPPORT SERVICES

Overview

DERC investigators can receive assistance with a variety of study design and analytic methods. Depending upon the prior experience investigators have with human genetics studies, they may wish assistance in planning studies from inception on, or may only request assistance with deciding which type of genetic analysis to apply to their data after collection. Analytic support is provided by Human Genetics Core personnel on an hourly consultative basis. Investigators have the option of requesting that analyses actually be performed by Core personnel as well as the option of receiving training in how to utilize the various genetic analysis programs themselves.
There are a variety of study designs that can be utilized for investigations into the genetic basis of diabetes and its complications. Choice of a specific design depends upon a number of factors, including the types of subjects available to the investigator, the specific phenotypes to be examined, and the hypotheses being tested. Core personnel can also provide assistance in estimating power and sample size, which vary depending upon whether a candidate gene or a systematic mapping approach is used, along with the degree of polymorphism of the DNA markers. The use of qualitative traits or quantitative traits and the methods of analysis selected will also influence power.

Consultation is available for a wide variety of genetic analyses, not just for analysis of genotyping data. For some studies, the first step may be to assess familiality, which gives an indication of the genetic determination of that trait. For genotyping data, assistance with both association studies and linkage analysis is available. The programs we are currently using include PEDCHECK, RELCHECK, PREST, RELTEST, RELPAIR, PEDRAW, LINKBASE, ACI, LOKI, PAP, PHYLIP, LINKAGE, S.A.G.E., MAPMAKER/SIBS, MENDEL, GENEHUNTER, CASPAR, ANALYZE, SIMWALK2, SIMLINK, FISHER, SOLAR, MEGA2, GOLD, QTDT, HAPLOVIEW, and PHASE.

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Procedure	Discount DERC Rate	# Procedures/ year/investigator at discount rate	Non-Discounted DERC Rate (Actual Cost)
Establish lymphoblastoid cell line/ freeze 5 pellets	$100.00	15	$150
Thaw existing lymphoblastoid cell line, regrow + create 5 pellets	$30.00	15	$ 50.00
Non-viable cell pellet from anonymized panel (~20 x 10^6 cells)	$15.00	15	$30.00
Viable cells in culture from anonymized panel (T-25 flask; ~25 x 10^6 cells)	$30.00	15	$50.00
Microsatellites, per genotype	$1.10	1000	$ 1.80
SNP setup	0	**	$850.00
SNP's, per genotype	$0.60	1250	$ 1.00
Genome scan, per genotype	$0.65	1250	$ 1.10
Customized high throughput SNP genotyping using the Illumina BeadStation (384 SNPs, 480 subjects)‡	N.A.	N.A.	$20,000.00
Bioinformatics consulting, hourly rate	$40.00	10 hrs.‡‡	$ 60.00
Statistical and/or study design consulting, hourly rate	$40.00	10 hrs.‡‡	$ 60.00

** SNP setup charge applies only when a specific SNP has not been run in the lab previously

‡ The first 10 set-ups in year 1, increasing to the first 20 set-ups in year 5, will not be charged to investigators. Quotations for other configurations (i.e. more specimens per run, different numbers of SNP's) on request.

‡‡ First hour of consultation per year will be at no charge.

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