Genetic surveillance of incident and prevalent HIV, HCV, and HBV infections in US blood donors

Protocol Synopsis

We propose to conduct a genetic analysis of incident and prevalent strains of HIV, HCV and HBV by testing blood specimens from HIV, HCV or HBV positive blood donors who give blood at REDS-II, UBS, BCP, NYBC and ARC blood centers between 2006 and 2008. The frequency of the different genetic lineages of these viruses will be evaluated and compared between incident (recently acquired) and prevalent (more remotely acquired) infections. We will also evaluate if genotypic distributions vary by demographic characteristics and by geographical area. Informative viral genetic regions will be PCR amplified, sequenced and phylogenetically analyzed to determine genotypes and subtypes. Anti-viral drug and antibody neutralization resistance mutations will also be recorded. This study will provide an oversight of the genetic diversity of blood borne agents detected in the US using current NAT, antigen and serological assays. Using published data, this analysis of viruses in blood donors will allow us to also determine whether this population is infected with the same strains of viruses as seen in more at-risk populations. Genetic characterization of viruses in NAT-yield and recent seroconverting donations, which represent incident cases, will allow for characterization of circulating strains of viruses thus enabling early detection of rare variants and longitudinal tracking of changes in genotype frequency of actively transmitted strains. Because genetic variants of HIV, HCV and HBV appear to differ in properties such as pathogenicity, anti-viral therapy response and antibody neutralization properties, the genetic characterization of incident strains will also guide future anti-viral and vaccine research in the US.

A. Background and Significance

Screening of blood donations for HIV and HCV RNA using nucleic acid amplification technologies [NAT] and DNA-NAT or surface antigen immunoassays (HBsAg) for HBV allows the identification of infectious donations prior to the development of anti-viral antibodies. HIV and HCV NAT assays are currently being used to screen the US blood supply and have considerably reduced transfusion-transmitted risks for these viruses (Offergeld et al., 2005; Stramer et al., 2004; Yoshikawa et al., 2005) (Busch et al., 2005) . The identification of donations from pre-seroconversion blood donors also provides a unique opportunity to genetically analyze viral strains that have been recently acquired and to compare them to prevalent strains in demographic groups considered at low risk for these infections (i.e., non-remunerated blood donors).

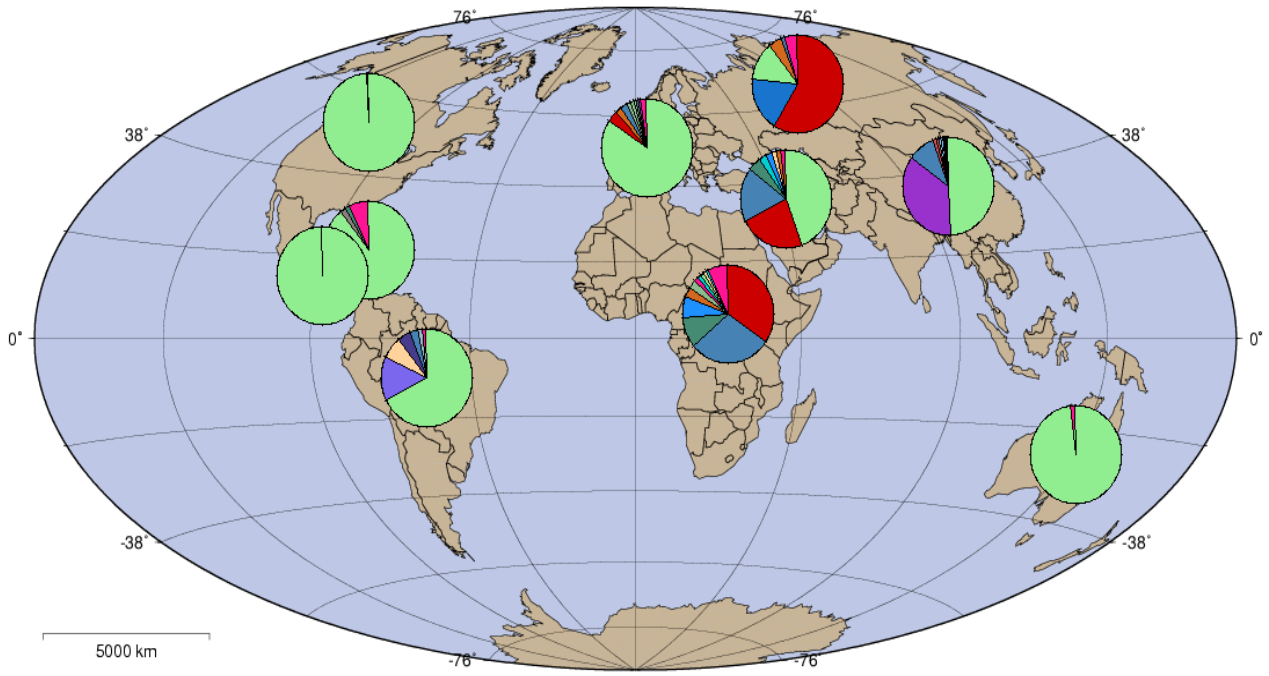
By comparing the distribution of variants belonging to different genetic lineages, sequence analysis of incident and prevalent viral strains allows the molecular epidemiology of these pathogens to be tracked over time and in different geographic areas. HIV, HCV and HBV consist of readily recognizable lineages whose relative frequency varies considerably over time and in different regions. The frequency of these distinct genetic lineages (also referred to as clades, genotypes or subtypes) reflects viral trafficking into and within risk groups and geographic regions. Infection with different viral lineages can also be clinically relevant as subtypes can affect disease progression rates. Detailed genetic analyses within each viral lineage can also identify variants that have reduced susceptibility to anti-viral or immunoglobulin therapies (HIV and HBV), as well as to neutralization by vaccination induced antibodies (HBV). Genetic variation may also reduce the sensitivity of serological, antigen or nucleic acid tests used for their detection and quantitation, of particular concern for the accuracy of blood and organ donor screening but also important with respect to use of these assays as diagnostic, prognostic and therapeutic monitoring tools.

The genetic variability of HIV, HCV and HBV has been the subject of numerous studies and has resulted in sequence databases that include bioinformatics tools for their analyses. The HIV sequence database can be analyzed at http://hiv-web.lanl.gov and http://hivdb.stanford.edu, HCV sequences at

http://hcv.lanl.gov, http://s2as02.genes.nig.ac.jp and http://euhcvdb.ibcp.fr/euHCVdb/ and HBV sequences at http://s2as02.genes.nig.ac.jp.

A1. Genetic diversity of HIV

Human lentiviruses consist of two viral species, HIV-1 and HIV-2. HIV-1 consists of three lineages that have been identified to date (group M, N, and O) (Alaeus, 2000; Holmes, 2001; Sharp et al., 2001). Each lineage is made up of numerous subtypes (clades) as well as recombinants of these subtypes. HIV-2, some strains of which are indistinguishable from SIVsm derived from sooty mangabeys (Marx et al., 1991), is also made up of several subtypes (Damond et al., 2004). The initial HIV-1 strain identified belonged to HIV-1 group M subtype B; this subtype is currently the most prevalent subtype in North America and Europe, whereas subtype C is most prevalent world-wide) (http://hiv-web.lanl.gov). For historical and practical reasons assays used to detect serological evidence of HIV infection were originally designed using HIV-1 group M subtype B virus. The subsequent identification of HIV-1 group M subtypes, HIV-1 groups N and O as well as HIV-2 has led to the requirement that the sensitivity and specificity of nucleic acid, serological, and antigenic assays be determined with respect to infections by divergent strains. When low cross-reactivity of existing tests to the new HIV variants was observed due to a high degree of genetic divergence, pre-existing tests were improved, replaced, or complemented with more sensitive tests (Apetrei et al., 1996b; Dondero, Hu, and George, 1994; Loussert-Ajaka et al., 1994; Schable et al., 1994b).



Distribution of major subtypes and CRF of HIV1

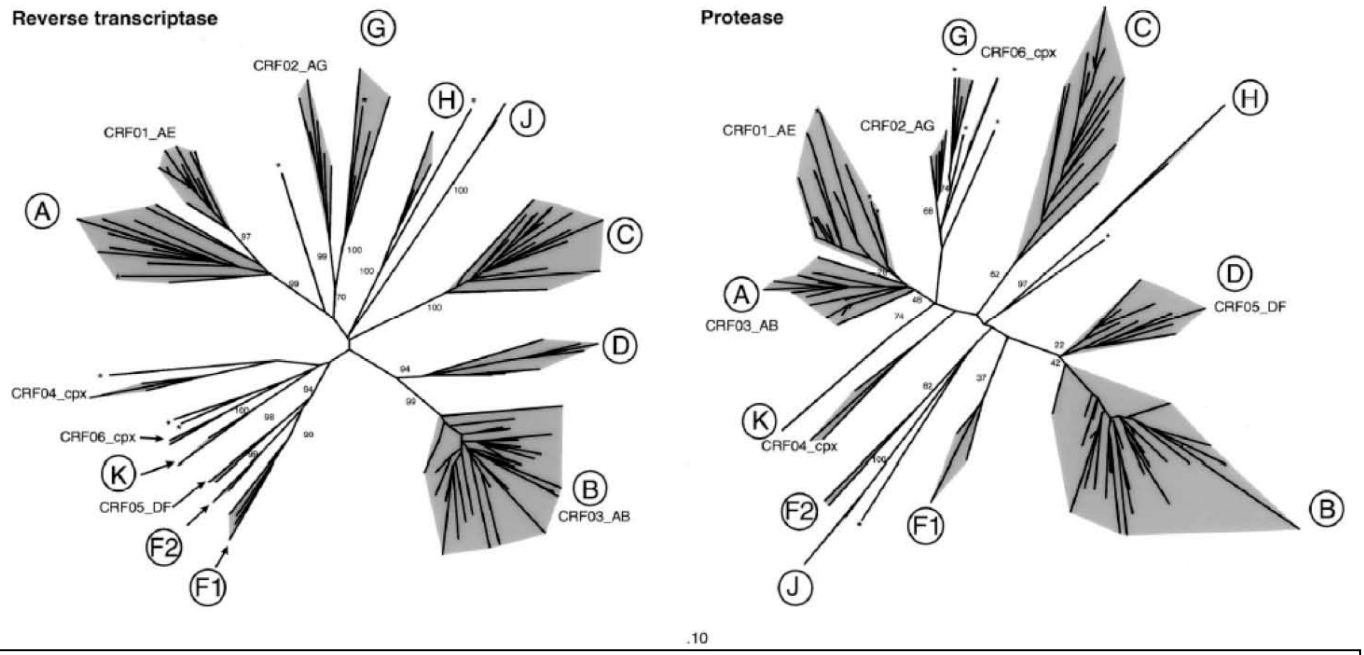
By 2005 over 11 subtypes of the M group of HIV-1 as well as 16 common recombinant forms had been identified (http://hiv-web.lanl.gov). The US remains one of the most genetically homogeneous regions in terms of HIV-1 diversity, with > 99% subtype B infections (http://hiv-web.lanl.gov). However an increasing number of HIV-1 subtypes have been detected in US blood donors (Bennett, 2005; Delwart et al., 2003; Sides et al., 2005) (Table 1). The increasing genetic diversity in the US is unlikely to lead in the *near* future to a situation similar to that in Europe where the multiple subtypes and recombinant forms of HIV-1 group M have been detected at high frequency along with HIV-1 group O (and HIV-2)

viruses (http://hiv-web.lanl.gov) (Fleury et al., 2003; Roques et al., 2002). Group N HIV-1 has been so far detected only in Eastern Africa. The largest degree of HIV-1 genetic diversity has been recorded in the democratic republic of Congo (Vidal et al., 2000).

Effect of HIV genetic diversity on EIA and viral loads measurements. While quantitative nucleic acid testing assays for HIV-1 targets a highly conserved region of HIV-1 group M, the large degree of genetic variability of this virus has been shown to affect the accuracy of viral load assays (Chew et al., 1999; Emery et al., 2000; Jenny-Avital and Beatrice, 2001). The impact of subtype genetic variation on EIA sensitivity has been less extensively studied due to the rarity of longitudinal panels collected during the seroconversion period from persons with non-subtype B primary infections (Phillips et al., 2000; Schable et al., 1994a). While licensed EIAs can clearly detect HIV-1 group M non-subtype B induced antibodies from chronically infected persons and are mandated to do so for FDA approval, their sensitivity during early seroconversion may be lower (Apetrei et al., 1996a). The importance of HIV molecular surveillance for the purpose of diagnostic, research and prevention has been reviewed (Hu et al., 1996).

HIV-2 screening of donors in the US remains serologically based due to the very low prevalence and incidence of the HIV-2 in this country. As for HIV-1 the presence of multiple subtypes of HIV-2 indicates that EIA sensitivity may not be equal for all genetic subtypes, particularly early following infection when antibody titers are expected to be at low titers and of low affinity.

Effect of HIV genetic diversity on anti-viral therapies. Resistance to anti-viral drugs is known to



Phylogenetic classification of HIV1 RT and protease loci.

rapidly evolve in vivo during therapy with sub-optimal drug regimens. Numerous studies have mapped the location of amino acid changing mutations in HIV-1 and correlated their presence with reduced viral susceptibility to protease and reverse transcriptase (RT) inhibitors. The exact location of non-synonymous (amino acid changing) HIV-1 mutation in the protease and RT genes therefore indicates which anti-viral drugs will have reduced activity against the mutated strain. Drug resistant strains can also be transmitted, and the frequency of primary infection with drug resistant strains in high-risk groups has been increasing since the beginning of anti-viral therapies starting with the 215Y mutation resulting in high-level resistance to AZT (Conlon et al., 1994; Little, 2000; Yerly et al., 2001). Web sites have been developed and are constantly updated where "virtual" drug resistant phenotypes can be derived

from HIV-1 sequence data (http://hivdb.stanford.edu and http://hiv-web.lanl.gov). In the US, the frequency of primary infections with drug resistant strains is estimated to be approximately 10% (Little et al 2002, Weinstock et al, 2004)

Prior studies of HIV infections in blood donors. A limited number of studies have used the large scale screening capacity of blood banks to identify prevalent and incident cases of HIV infection in blood donors for further studies of molecular diversity. The prevalence of non-B subtypes in infected U.S. blood donors was found to be 2% during 1997-2000 (de Oliveira et al., 2000; Delwart et al., 2003) and appeared to be increasing (Table A.1).

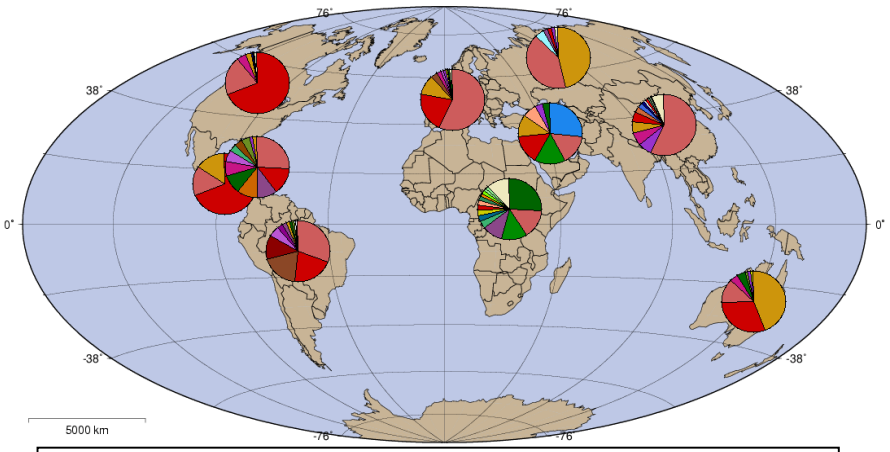
Table A.1. Frequency of non-B HIV-1 subtypes among HIV-1 viremic donors in the U.S. donor pool.

Study Period	Population	# studied	# (%) Non-B HIV1 Subtype	
'84-'85	TSS donors & hemophilliacs	143	0	
'93-'96	Donors in CDC study	383	2 (0.8%)	1 C, 1 CRF A/G
'97-'98	Donors in CDC study	163	3 (1.8%)	3 Cs, 1 HIV-2
'99-2000	Donors in CDC study	130	4 (3.1%)	2 C, 1CRF01, 1A

Using the detuned EIA strategy to identify recent infections, non-subtype B and drug resistance mutations were also detected among HIV-1 strains that had been recently transmitted to US blood donors (Machado et al., 2002). The frequency of different HIV-1 subtypes among recently infected donors identified from detuned EIA testing has also been estimated in Brazil (Morgado et al., 1994). Panels of longitudinally collected plasma from seroconverting plasma donors have been valuable for detailed studies of the diversity within the transmitted viral HIV population (Delwart et al., 2002) and of its subsequent evolution within subjects (Bernardin et al., 2005). Longitudinal plasma panels have also revealed the presence of very low level HIV, only detectable by repeated NAT testing, prior to the emergence of chronic higher level viremia (Fiebig et al., 2005). The frequency of transfusion mediated HIV-1 transmission has been estimated using the incidence rate deduced from the rate of NAT positive but serologically non-reactive donations (Busch et al., 2005). Transfusion-transmission of HIV from pre-seroconversion donations with very low HIV-1 viral loads have been confirmed using phylogenetic analyses of the donors and recipient viruses (Delwart et al., 2004; Ling et al., 2000). These findings emphasize the importance of monitoring the genetic diversity of HIV-1 in the population of donors with recently acquired infections.

A2. Genetic diversity of HCV

The genetic diversity of HCV is considerably greater than that of HIV-1 group M, possibly reflecting the older nature of HCV in humans. For example, the genetic distance between all HIV-1 group M subtypes is similar to that measured within a single genotype of HCV. Six genotypes of HCV viruses have been identified to date, each containing multiple subtypes whose geographic distributions differ greatly. Over 90% of HCV infections in the US are of genotype 1 (69.8% 1a, 20.6% 1b) with other

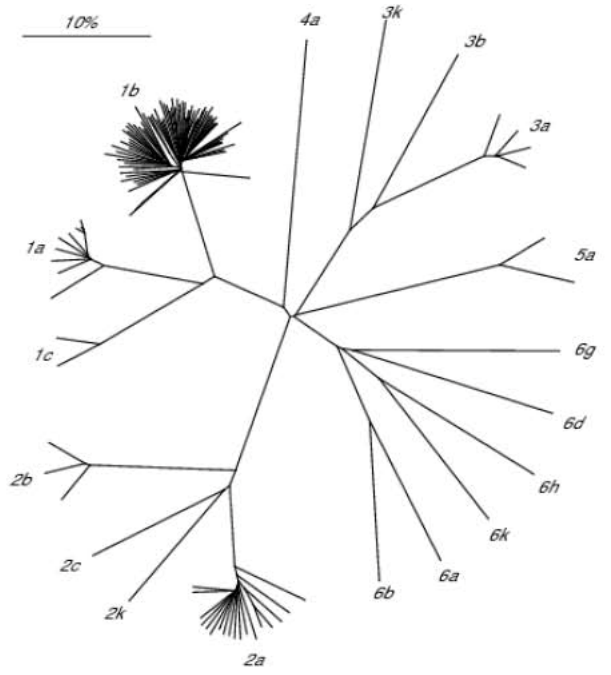


Distribution of HCV genotypes and subtypes

genotypes less frequently seen (5.1% 2b, 3.3% 3a, 1.3% others- http://hcv.lanl.gov/). Only a single case of a recombinant genotype has been reported for HCV (Kalinina, Norder, and Magnius, 2004). Because of its large degree of genetic divergence, HCV genotypes and subtypes can be readily identified by phylogenetic analysis using small regions of the viral genome.

Effect of HCV genetic diversity on EIA and viral loads measurements. The effect of HCV sequence variation on NAT tests has not been as extensively tested as for HIV-1. In one multicenter study, genotypes 1 and 3 were detected with equal sensitivity to TMA and PCR assays (Lelie et al., 2002), while other assay specific studies showed comparable detection of all subtypes tested (Saldanha et al., 2003; Vargo et al., 2002).

Effect of HCV genetic diversity on response to anti-viral therapies. Recent progress in HCV treatment includes use of long half-life pegylated alpha 2A interferon and ribavirin combination therapies. Early reports of HCV sequence data in non-responders and people with only a short-term control of viremia reported a possible association with certain mutations in the NS5B region, but these reports were not confirmed in later studies (Chambers et al., 2005; Nousbaum et al., 2000; Polyak et al., 2000). Many compounds targeting the HCV protease, helicase and polymerase are being developed (Bartenschlager, 1997). Analysis of mutations selected by these compounds using sub-genomic replicons in tissue culture have identified resistance mutations located in the genetic regions encoding the expected target proteins. Because none of the viral enzyme targeting compounds are currently in use, detection of these resistance mutations in the population is not currently expected.



Phylogenetic analysis of HCV genomes.

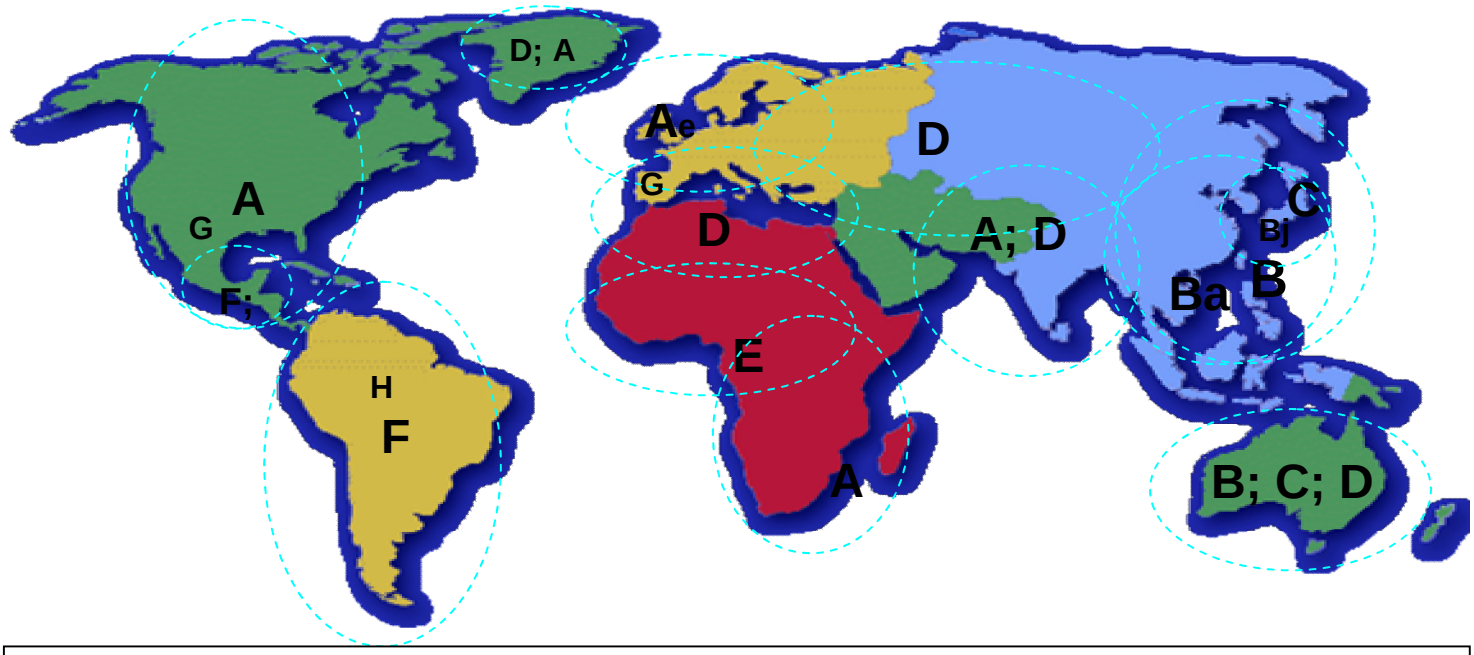
Subtypes are also known to influence the rate of successful control of HCV viremia following IFN treatment. HCV genotype 1, the most common in the US, has a demonstrably lower rate of response to therapy than the other genotypes (McHutchison et al., 1998; Zein et al., 1996).

Studies of HCV infections in blood donors. HCV subtype distribution has been analyzed in HCV NAT positive donation collected over the past several years. A significant shift in the dominant genotypes has been observed (S. Stramer, personal communications). Similar studies of HCV and HBV in Japan has shown that different distributions of genotypes present in donors versus hospitalized patients (Murokawa et al., 2005).

A3. Genetic diversity of HBV

Eight genotypes of HBV have been identified in humans (Kidd-Ljunggren, Miyakawa, and Kidd, 2002). There is evidence that infection with genotype D results in higher ALT levels (Kidd-Ljunggren, Miyakawa, and Kidd, 2002; Kidd-Ljunggren, Myhre, and Blackberg, 2004). The geographic distribution of the genotypes varies considerably. Genotype A is mainly found in Northern and Central Europe, North America, and Australia, although some strains have also been found in the Philippines and South and Eastern Africa. Genotype B and C are common in Asia, but are also found in North America and Australia. Genotype D has the widest geographic distribution. Although it has been found universally, it is especially common in the Mediterranean region and stretching from Southern Europe and North

Africa to India, in West and South Africa, as well as in Australia. Genotype E, genetically similar to D, is found in West and South Africa. Genotype F, the most divergent genotype, is found in South and Central America. Genotype G is found in France and the United States. Genotype H is the most recently identified genotype and is found in Central America. In the United States, a recent study found genotypes A and C to be the most common (37.4% A, 30.8% C, 22% B, 10.4% D, 0.4% E, 0.6% F, 1.1% G) (Chu et al., 2003). Furthermore, a strong correlation was found between HBV genotypes and ethnicity: genotypes A and D were most common in Caucasians and genotypes B and C were most common in Asians (Chu et al., 2003).



Geographic distribution of HBV subtypes

Effect of HBV genetic diversity on EIA and viral loads measurements. Reduced detection of HBV infection using HBsAg has been reported to be affected by mutations in the surface glycoprotein (Jongerius et al., 1998). Because viral loads measured by HBV DNA content are increasingly being used to monitor response to anti-viral therapies on disease progression, the effect of genotypic variation on the accuracy of these assays may be of clinical relevance. Unfortunately there are no large scale studies published looking at this issue.

Effect of HBV genetic diversity on anti-viral therapies and antibody neutralization: The oldest therapy for advanced HBV infection (and consequent liver disease) is interferon alpha, although toxicity and low response rates are problematic. Multiple anti-viral drugs are now also in use targeting the HBV polymerase. Lamivudine selects mutants in the YMDD active site while adefovir select mutations close downstream of that motif (N236T and A181V) (Nafa et al., 2000). Lamivudine is known to select resistant mutants in ~60% of treated patients within 3 years.

Mutations associated with decreased sensitivity to neutralization by anti-HBs have also been identified in the gene encoding the surface glycoprotein. For example a common mutation G145R leads to complete escape from neutralization, and this strain is frequently found in vaccinated persons who still became HBV infected and long-term HBV carriers. Immunoprophylaxis of HBV at birth or following liver transplantation can also select HBV strains resistant to hyperimmune IgG therapy. In Spain 9.2% of HBV infections are HBsAG mutants resistant to immunotherapy and 6.6% are vaccine escape mutants (Schochetman, Gerlich, and Kuhns, 2005).

B. Objectives

B.1 Primary objectives

a. Measure the frequency of distinct viral lineages in incident and prevalent blood donor infections. We will measure the frequency of different genetic clade/genotype/subtype in prevalent and recently acquired infections; and

b. Measure frequency of anti-viral drug resistance mutations in HIV and HBV (plus neutralization resistance mutations for HBV) in incident and prevalent blood donor infections.

B.2 Secondary objectives

a. Compare the frequencies of different lineages and drug and neutralization resistant variants between incident and prevalent cases;

b. Evaluate whether the frequencies of different lineages and drug and neutralization resistant variants in incident and prevalent cases vary by demographics (age, gender, and if available race/ethnicity) and by geographical location;

c. Evaluate whether the frequencies of different lineages and drug and neutralization resistant variants in incident cases changes with time (2006-2008);

d. Compare variant frequencies documented in infected donors to those in high-risk groups reported in the literature; and

e. Using residual volume from the characterized specimens, create a linked repository for future HIV, HBV, and/or HCV studies.

C. Overview of proposed study

This study is proposed as a 3-year genetic surveillance analysis of the strains of HIV, HCV and HBV in recently infected blood donors. Further, genotypic analysis of first-time prevalent cases will be conducted to allow for insights into changes in viral diversity over time.

Between January 2006 and December 2008, laboratories that conduct HIV, HCV and HBV screening/confirmatory testing of all donations collected by the UBS/BCP blood centers, the NYBC, the ARC blood centers and the 3 REDS-II blood centers not belonging to ARC or BSL, will identify all donations that meet the criteria of incident HIV, HBV or HCV infection (Section D.1) and will send corresponding specimens to the REDS-II central laboratory (BSRI) for molecular sequencing of the appropriate viral regions. The laboratories will also identify all HIV antibody-confirmed positive donations and send corresponding specimens to BSRI for evaluation by the detuned EIA assay as a supplemental method to identify recently acquired HIV infections (occurring within the last six months).

The participating laboratories will also identify prevalent infections (Section D.2) occurring at these centers and will send corresponding samples to BSRI for testing. Prevalent infections will be defined as first-time antibody-positive donations (for HIV, these would be the antibody-positive donations that occurred >6 months prior to the index donation per the detuned EIA assay). The same number of prevalent and incident cases (collected during 2006-2008) will be genotypically analyzed (estimated at

150 each per virus). It is expected that a sufficient number of first-time prevalent HCV and HBV positive donations will be identified during the first 4-6 months of this study in 2006 while it will be necessary to identify HIV first-time prevalent infections over a longer period of time (≈2 years).

Participating organizations (REDS-II, ARC, UBS/BCP, NYBC) and/or laboratories will also provide to the coordinating center (Westat) information on the incident and prevalent cases' demographics (age, gender, race/ethnicity if available, first time and repeat status and 3 digit zip code) and summary statistics on the total number of allogeneic donations screened as well as their demographic breakdown (age, gender, race/ethnicity if available, first time and repeat status, and possibly geographic area). The coordinating center will compile the incident and prevalent cases' demographic data with corresponding genotypic test result information provided by the REDS-II central laboratory. The resulting dataset will be used for statistical analysis. Summary statistic data will be used to describe the population screened and to calculate overall rates, as warranted.

The REDS-II central laboratory will extract DNA and RNA and perform RT-nested PCR using appropriate PCR primers for fragments of HIV and HCV. Nested PCR (i.e. without RT step) will be used for amplification of HBV DNA. The large genetic diversity of HCV and HBV will require the sequential use of different primer sets should the initial attempts not yield PCR products. PCR products will be purified and directly sequenced. Sequence data will be proof-read for quality and their sequences assembled and entered into a final database. Viral sequences will then be analyzed using different web sites in order to determine a) phylogenetic clade/subtype for HIV, HCV and HBV, b) drug resistance genotype for HIV and HBV and c) resistance to neutralization for HBV.

Sero-prevalent cases of these 3 viruses will be similarly analyzed. These analyses will be performed to compare the currently transmitted strains of virus in seronegative NAT-positive donors (in addition, for HIV, the seropositive donations that are identified as occurring within the last 6 months by the detuned EIA assay will be included as incident cases) to those in "older" sero-positive infections (for HIV, the seropositive donations that are identified as occurring more than 6 months ago by the detuned EIA assay) in order to detect a change in the composition of the viral pool infecting blood donors. We will also evaluate if genotypic distributions vary by demographics and geographical area, and whether changes occur among incident donors with time. Data from these blood donor molecular surveillance studies will also be compared to pre-existing published data in US blood donors and to other sources of data outside the blood donor screening setting.

Finally, residual plasma volume of the samples analyzed in this study will be retained in a small linked repository. Small repositories of such difficult to assemble, characterized samples are an invaluable resource and will allow for additional studies related to HIV, HBV, and HCV. Future studies conducted using samples from this repository may or may not be anonymized and approvals from both the OSMB and IRBs will be sought before further studies on these samples are initiated.

D. Study population

Specimens from HIV, HCV or HBV-positive blood donors will be obtained from the central laboratories of the two major blood collection systems in the US (ARC and BSL- the latter includes UBS, BCP and the NYBC) who have agreed to collaborate on this project and from the 3 REDS-II centers that are not part of these organizations. Because demographic data are not routinely compiled in a database for all of BSL, a subset of centers for which a donation database is readily available will participate (UBS, BCP and the New York Blood Center); these centers together contribute about 1.8 million donations annually. In total, the REDS-II centers and both collaborating organizations will contribute information on about 8 million donations annually, estimated to account for 70% of the US blood supply (Appendix I). We anticipate receiving for genetic analyses up to 50 cases of incident infection for each virus per

year, for a total of up to 150 incident infections per virus over the three year study period (Appendix I). We will also analyze a similar number of prevalent HIV (identified over ≈ 2 years) and HCV and HBV infections (identified in 4-6 months in 2006).

Specimens will consist of the residual volume obtained from index donation tubes that were originally collected for NAT screening or for serological testing. All HIV, HCV and HBV positive specimens identified as of January 2006 will be saved by participating laboratories to enable retrospective testing of specimens collected early in 2006 once the study has been approved by all appropriate IRBs and NHLBI.

D.1 Incident infections in US blood donors: Definitions

Incident cases of HIV, HCV and HBV will be identified during 2006-2008 by the laboratories that screen all donations collected by ARC, UBS/BCP, the NYBC, and the three REDS-II blood centers that are not part of ARC or BSL.

HIV-1
Incident infections will be defined as those that are RNA positive and HIV-antibody negative during routine blood screening. However, it is anticipated that only ~5-10 such infections will be detected by the participating laboratories per year. In order to derive a larger and more representative sample of recent HIV infections in blood donors we will also include donation samples with low signal in a detuned EIA using the Standardized Testing Algorithm for Recent HIV Seroconversion (STARHS) (Janssen et al., 1998; Machado et al., 2002; McFarland et al., 1999). Aliquots from all HIV-confirmed antibody positive samples will be saved and sent to BSRI (the REDS-II central laboratory) for testing by DT/LS EIA to evaluate eligibility for the study. A STARHS standardized optical density of <0.75 will be used to identify seropositive infections that have occurred within the last 6 months. Using this criterion, we estimate that 40-50 additional incident strains of HIV will be collected per year (Appendix I) since we estimate that approximately 25% of HIV antibody-positive donations will be recent infections (Busch et al., 2005). The RNA viral loads of each sample will be determined in order to ensure that sufficient virus is present to ensure PCR amplification. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using standard HIV PCR primers).

HCV
Incident infections can be identified as those that are RNA positive and HCV-antibody negative during routine blood screening. Approximately 25-50 seronegative incident HCV infections are expected to be identified yearly by the participating laboratories (Appendix-I). The RNA viral load of each sample will be determined in order to ensure that sufficient virus is present to ensure PCR amplification. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using standard HCV PCR primers).

HBV
Incident infections will be identified as HBsAg positive donations that are anti-hepatitis B core antigen (anti-HBc) non-reactive. In the future, HBV NAT reactive, HBsAg negative, anti-HBc negative donations will also be included. It is estimated that 50 or more samples will be detected per year by the participating laboratories (Appendix-I). The DNA viral load of each sample will be determined in order to ensure that sufficient virus is present to ensure PCR amplification. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using HBV PCR primers).

D.2 Prevalent infections in US blood donors: Definitions

The prevalent cases of HIV, HCV and HBV infection included in the study will be derived from all seropositive first-time donations (for HIV, those antibody-positive donations that occurred more than 6 months ago per the detuned EIA assay) collected by participating centers in 2006. A donation will be considered first-time if it represents the first donation given by the donor at that center since June 1996 when the more sensitive third generation EIA test for HCV was implemented. We would like to accrue for each virus a total of 250 antibody-positive first-time donations to be able to test 150 with sufficient viral load. It is estimated that a period of about 4-6 months beginning in Jan 2006 will be sufficient to provide a sufficient number of prevalent infection samples for HCV and HBV while accrual of first-time HIV prevalent cases will need to occur over ≈2 years to permit identification of a sufficient number of specimens.

HIV-1

Prevalent infections will be defined as those NAT-positive antibody-positive first-time donations that have a signal to cutoff signal ratio in a detuned EIA using the STARHS algorithm (Janssen et al., 1998; Machado et al., 2002; McFarland et al., 1999) indicating that the infection was acquired more than 6 months ago. About 75% of all HIV antibody-positive donations will probably fall in this category (Busch et al., 2005). We plan on testing the same number of prevalent samples as incident samples (n≈150). The RNA viral loads of up to 250 samples will be determined (using Chiron or NGI assays) in order to ensure that sufficient virus is present to ensure PCR amplification in about 150 samples. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using standard HIV PCR primers).

HCV

A number of prevalent first-time HCV infections (defined as RNA NAT-positive, HCV antibody-positive units) identical to that of incident cases (estimated to number 25-50 cases per year or 800-150 over the three year study period) will be selected. Prevalent cases will be derived from the first 250 cases identified proportionately by the participating centers in 2006. The RNA viral load of each sample will be determined using either the Chiron or NGI quantitative assay in order to ensure that sufficient virus is present to ensure PCR amplification. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using standard HCV PCR primers). A total of about 150 prevalent cases will be randomly selected from qualified donations and genotypically analyzed.

HBV

Prevalent infections will be defined as anti-hepatitis B core antigen (anti-HBc) reactive and HBsAg-positive donations. 250 prevalent samples will be selected by the participating laboratories to ensure testing of about 150. The DNA viral load of each sample will be determined using the Chiron or NGI quantitative assay in order to ensure that sufficient virus is present to ensure PCR amplification. A viral load (VL) of >500 copies per ml will be considered sufficient for genetic analysis (i.e. high enough to amplify using HBV PCR primers).

Table D. Summary of the characteristics of the HIV, HCV and HBV positive specimens that will be evaluated:

Confirmatory Test Result	Definition for the Study
HIV	
HIV Ab-neg + HIV NAT-pos	Incident (recent infection)
HIV Ab- pos (detuned assay indicates recent infection) + HIV NAT-pos	Incident (recent infection)
HIV Ab- pos (detuned assay indicates remote infection) + HIV NAT-pos	Prevalent (remote infection)
HCV	
HCV Ab-neg + HCV NAT-pos	Incident (recent infection)
HCV Ab- pos + HCV NAT-pos	Prevalent (remote infection)
HBV	
HBsAg-positive + Anti-HBc non-reactive	Incident (recent infection)
HBsAg-positive + Anti-HBc reactive	Prevalent (remote infection)

E. Study design

E.1 Identification of Incident and Prevalent Cases and Tracking of Specimen Information

The flow diagram presented in Appendix-II summarizes the proposed study design. Between January 2006 and December 2008, laboratories that conduct HIV, HCV and HBV screening/confirmatory testing of all donations collected by the UBS/BCP blood centers, the NYBC, the ARC blood centers and the 3 REDS-II blood centers not belonging to ARC or BSL, will identify all donations that meet the criteria of incident HIV, HBV, or HCV infection (see section D.1) and will store at $\leq -20^0$C residual plasma from the corresponding NAT and/or serological tubes. These donation specimens will then be shipped to the REDS-II central laboratory (BSRI) every 5-6 months for molecular sequencing (after IRBs have approved the study). The laboratories will also identify all HIV antibody-confirmed positive donations, store the corresponding residual tubes, and ship every 5-6 months the corresponding specimens to BSRI for evaluation by the detuned EIA assay.

The participating laboratories will also identify prevalent infections (see section D.2) occurring at these centers and store plasma from the corresponding NAT and/or serological tubes at $\leq -20^0$C. The same number of prevalent and incident cases will be genotypically analyzed. It is expected that the first half of 2006 (January-June 2006) will probably yield a sufficient number of HCV and HBV prevalent infections for this study and that shipping of these samples to BSRI for testing will immediately follow IRB approvals of the study. For HIV, all HIV antibody-confirmed positive donation specimens will be sent to BSRI for evaluation by the detuned EIA assay. The first-time HIV-positive donations for which the detuned EIA indicates that the infection was acquired more than 6 months will be identified as prevalent cases. We anticipate that identification of a sufficient number of first-time HIV prevalent specimens will take about 2 years.

All specimen shipments to BSRI (one in September 2006 for HCV and HBV prevalent cases; every 5 to 6 months for approximately 3 years for incident and HIV antibody-positive specimens starting September 2006) will be tracked using the STS (Westat's Specimen Tracking System customized for REDS-II studies) and will be accompanied by a hard-copy list of all specimens' blood unit numbers or BUIs; if the BUI does not contain information on the center of origin, that information will be captured as a separate variable linked to the corresponding BUI. The same list (eg, an Excel file) will be concurrently emailed to BSRI. BSRI will use the STS to trak the shipments and receipts of specimens from the laboratories.

BSRI will conduct all laboratory testing (Section E.5) and link all test results and final interpretations to the appropriate specimen using BUI. The test result dataset compiled by BSRI will contain the genotypic results and final interpretations for each of the BUI labeled samples. BSRI will send this dataset as an Excel file to the REDS-II coordinating center (Westat).

E.2 Specimen Type, Volume and Storage Conditions

The tubes labeled with the BUI containing the residual plasma volume from NAT testing (HIV and HCV) or HBsAg testing, will be stored by participating centers at $\leq$-20^{0}C, preferably at –80^{0}C. The plasma (or serum) specimens' minimal acceptable volume will be 1.0 ml. Batch shipments to BSRI will be on dry ice and occur every 5-6 months.

E.3 Development of a Small Linked NHLBI Special Repository

After testing for this study is completed, residual plasma will be placed in –80^{0}C freezers in a repository. The repository specimen vials will be labeled with a newly assigned sample-ID only; thus, the labels will not consist of the BUI or other center-assigned IDs. The link between the sample-ID and BUI will be maintained in the password protected REDS-II specimen tracking system (STS). This small repository will be compiled by BSRI during the study period and be shipped to the NHLBI repository contractor (Seracare) once the study is completed i.e., by the end of the current contract.

E.4 Collection of Demographic and Geographical Information from Participating Organizations

When specimens are shipped to BSRI, participating organizations (ARC, UBS/BCP, NYBC) and/or laboratories will also email a file to Westat that contains screening test and demographic information on all incident HIV, HCV, HBV positive donations and on all HIV-antibody positive donations identified during the preceding 6 month period. This file will also indicate which of these positive donations had specimens that were shipped to BSRI. A similar file containing information for all HCV and HBV prevalent cases identified in the first 4-6 months of 2006 will be sent to Westat when the corresponding shipment to BSRI is made. The files sent to Westat will include: 1) information on donation date, BUI, center (if not included in the BUI), viral confirmatory test results (overall interpretation); 2) whether sufficient residual volume existed and a specimen was shipped to BSRI; and 3) corresponding information on age (date of birth), gender, 3-digit zip code and, if available, race/ethnicity.

The participating organizations will also provide Westat with *yearly* summary statistical files that will include the total number of allogeneic donations screened during the corresponding year (2006-2008) and the demographic distribution of allogeneic donations (age, gender, race/ethnicity if available, first time and repeat status, and possibly geographic area). While the REDS-II centers/laboratories will need to provide Westat with a file containing information on center, donation date, BUI, and HIV, HCV and HBV overall test result interpretation for those specimens that were shipped to BSRI, the case-specific demographic information and denominator data information for the 3 REDS-II centers will be directly extracted by Westat from the REDS-II donation database. Westat will be responsible for compiling laboratory test information provided by BSRI to corresponding demographic data (see Section G) and for conducting the statistical analyses (Section H).

E.5 Laboratory Testing

a. Extraction of nucleic acids

We will use the combined DNA/RNA viral extraction kits from Qiagen for extraction of all nucleic acids from plasma samples. Proteins are denatured and viral nucleic acid release from particles using a lysis solution. The nucleic acid are retained on a silica-gel filter surface and released with water in a 40ul volume.

b. Generic reverse transcription and PCR reagents:

SuperScript One-Step RT-PCR Kit with Platinum Taq for RNA viruses (HIV and HBV):
Manufacturer: Invitrogen, Cat. no. 10928-042 (1-800-828-6686)
Kit includes: 500µl of 5mM Magnesium Sulfate, 1.25 ml of 2x Reaction Mix, 100µl RT/Platinum Taq Mix
Storage: -20°C freezer, PCR clean room H1537H
Use: Reverse transcription of HIV and HCV RNA and first-round PCR (RT-PCR)

dNTPs:
Manufacturer: PE (or Pharmacia BioTech), Cat # 27-2035-02 202501 for dUTP's
Stock Solution: 100 mM
WorkingSolution: 25 mM dNTPs: Mix 100µl each of dATP, dCTP, and dGTP, and then 50µl each of dTTP and dUTP. Aliquot 100µl of the mix into 0.5mL tubes
Store: -20°C or lower, freezer in PCR clean room H1537H or -70°C freezer in H1537A
Use: First and Second Round PCR master mix solutions

Amplitaq Polymerase Kit:
Manufacturer: Applied Biosystems, #N808-0167
Kit includes: TAQ DNA polymerase with buffer and magnesium chloride
Preparation: use as provided
Storage: -20°C or lower, PCR clean room H1537H
Use: Second round PCR

c. HIV

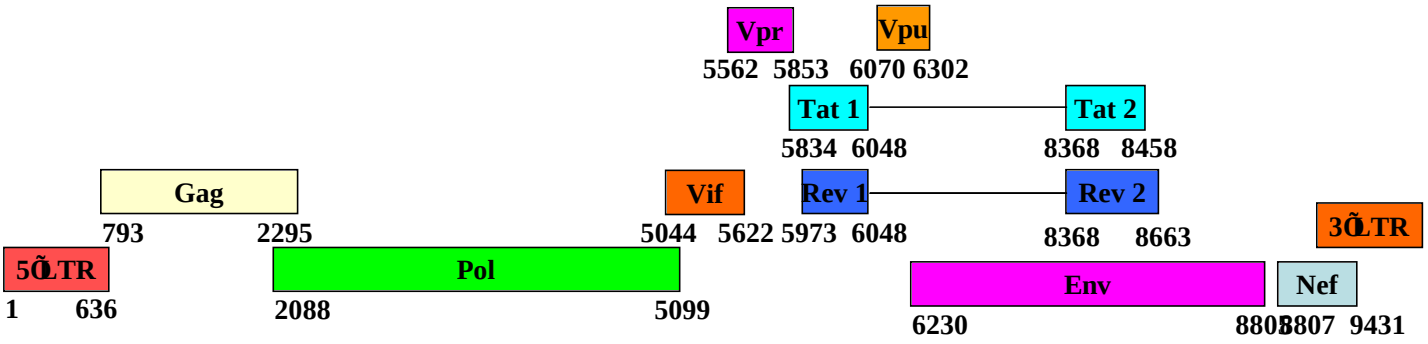
Nested PCR primers:
First and Second Round PCR Primers
Optimal primers were designed based on sequence alignments of HIV-1 from all subtypes. The most conserved regions were used to design primers particularly at the 3' termini of PCR primers. Nucleotide position with significant variability are handled using mixed bases in the primer shown here with the IUB code letter.

Manufacturer: Operon Technologies
Primers (5' to 3'): (HPLC purified)

First round:
MAW-26 TTggAAATgTggAAAggAAggAC
RT21 CTgTATTTCTgCTATTAAgTCTTTTgATggg

Second round:
PRO-1 CAgAgCCAACAgCCCCACCA
RT20 CTgCCAgTTCTAgCTCTgCTTC

Preparation: Rehydrate lyophilized powder by adding [#pmoles/50] µl of ultra-pure water.
Storage -20°C or lower, freezer in PCR clean room
Use: Component of RT and PCR reaction mixes



Genetic map of HIV RNA genome showing different ORF

Description of region amplified
For HIV we will amplify the entire protease gene (297 nucleotides encoding 99 amino acids) and the polymerase gene encoding the first 320 amino acids of the HIV RT enzyme. Drug resistance mutation conferring resistance to both protease and nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) have been mapped within this region. An amplification products will be generated which will be directly sequenced using six other primers.

Sequencing Reaction Primers

HXB2-89	AATCTgACTTgCCCAATTCAATTT
RT-Y	gTgTCTCATTgTTTATACTAg g
RT-A	gTTgACTCAgATTggTTgCAC
RT-B	CCTAgTATAAACAATgAgACAC
MAW46	TCCCTCAgATCACTCTTTggCAACgAC
DSPR	gggCCATCCATTCCTggC

Data assembly and analysis
The raw sequence data will be proof-red and assembled into a single sequence using DNAStar software. The sequence will then be entered into the Stanford University drug resistance database web-site at: http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm in order to determine if mutations known to convey drug resistance are present.
In order to subtype the HIV present in blood donors the same sequence will also be entered in the NCBI web site at: http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi.
The HIV sequence analysis will therefore determine the frequency of prevalent and incident HIV strains in blood donors that are of non-B subtypes (currently the pre-dominant subtype in the US) as well as the frequency of strains that carry one or more high level drug resistance mutations. Drug resistance genotypes will therefore be generated automatically in the course of this genetic analysis and will not require additional experiments or expenses.

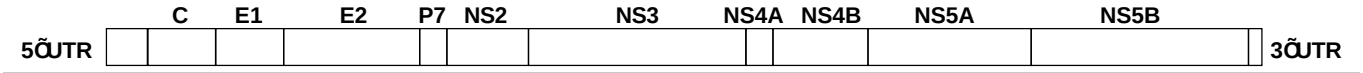
d. HCV
First and Second Round PCR Primers
Optimal primers were designed based on sequence alignments of HCV from all genotypes. The most conserved regions were used to design primers particularly at the 3' termini of PCR primers. Nucleotide

position with significant variability are handled using mixed bases in the primer shown here with the IUB code letter.

Manufacturer: Operon Technologies
Primers (5' to 3'): (HPLC purified)

First round:
HCV-S-1 TTGTGGTACTGCCTGATAGGG
HCV-AS-1 ATAGARAAAGAGCAACCKGG

Second round:
HCV-S-2 GCTTGCGAGTGCCCCGGGAGG
HCV-AS-2 GAGCAACCRGGHARRTTCCC



Genetic map of HCV RNA genome showing different ORF in polyprotein

Description of region amplified
These primers will amplify the core (C) gene of HCV that encodes the most conserved protein of HCV (facilitating use of common primers for different genotypes) while still containing sufficient diversity to identify HCV genotypes and subtypes.

Sequencing Reaction Primers
The amplicon will be sequenced using the second round PCR primers.

Data assembly and analysis
The raw sequence data will be proof-red and assembled into a single sequence using DNAStar software. HCV will then be genotyped using http://hcv.lanl.gov/content/hcv-db/BASIC_BLAST/basic_blast.html which uses BLAST similarity search against previously genotyped sequence in the Los Alamos Natl. Lab. HCV database.
The HCV sequence analysis will therefore determine the frequency of the six different HCV genotypes in both prevalent and incident infections. Phylogenetic analysis will also allow a more detailed determination of the subtypes (currently 18 confirmed plus 58 provisional subtypes) within the six genotypes.

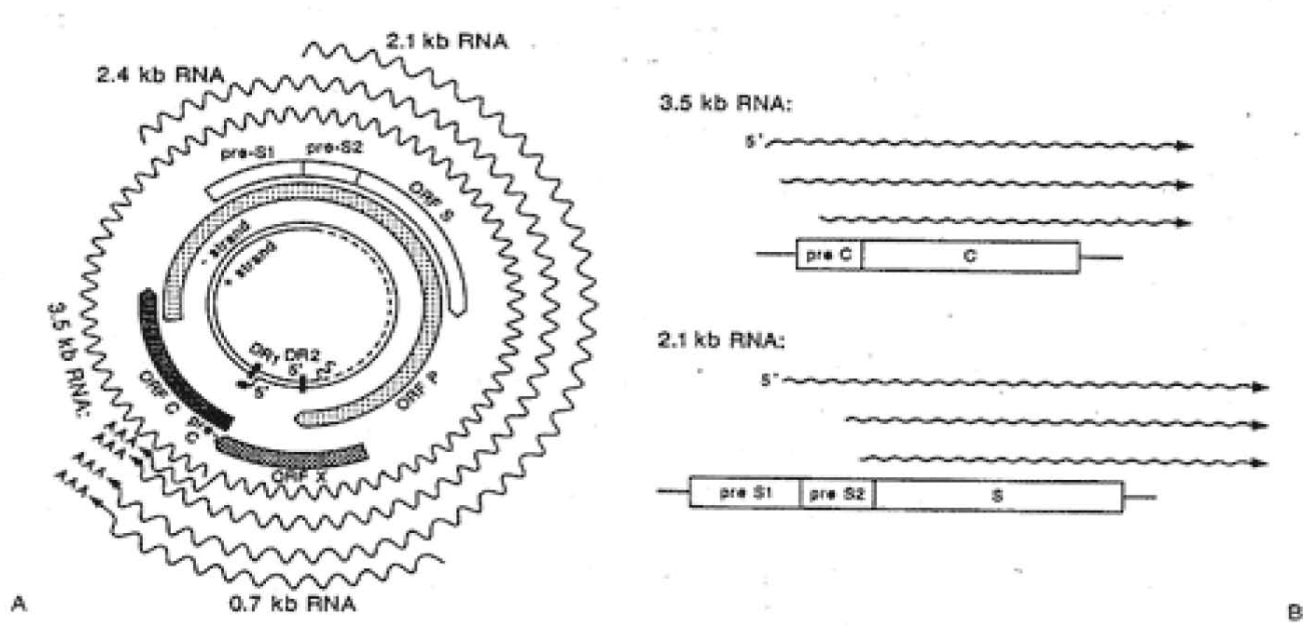
e. HBV
First and Second Round PCR Primers (no RT step necessary for HBV)

Manufacturer: Operon Technologies
Primers (5' to 3'): (HPLC purified)

First round:
HBVP1 T**KY**TGGGGTGGAGCCCTCAG
HBVP2 GAGTTCCGCAGTATGGATCG

Second round:
HBVP3 CCTGCTGGTGGCTCCAGTTC
HBVP4 TGC**R**TCAGCAAACACTTGGC

Description of region amplified
These primers will amplify the entire Surface (S) gene. Mutations preventing viral neutralization by antibodies raised during HBV vaccination have all been mapped to this region of HBV. The pre-S1 and pre-S2 ORF will not be amplified. On the other strand of the same amplified and sequenced region is encoded a region of the polymerase (P) gene. In this region of P have been mapped all HBV anti-viral drug resistance mutations.



Genetic map of HBV and location of its transcripts

Sequencing Reaction Primers:
Amplicon sequencing will be performed using the second round PCR primers.

Data assembly and analysis:
The raw sequence data will be proof-red and assembled into a single sequence using DNAStar software. HBV will then be genotyped using sequence similarity searches available at http://s2as02.genes.nig.ac.jp. The presence of anti-viral drug resistance mutations will be determined by translation of the P nucleotide sequence into an amino acid sequence and visual inspection for the presence of known resistance mutations. Lamivudine selects mutants in the YMDD active site while adefovir select mutations within 30 amino acids downstream of that motif (Nafa et al., 2000). Lamivudine is know to select resistant mutants in ~60% of treated patients within 3 years. The presence of mutations known to reduce susceptibility to antibody neutralization (most prominently G145R) will be similarly determined after translation of the sequenced region.

F. Statistical Considerations

F.1. Precision of estimated proportions in prevalent or incident infections:
The primary objective of this study is to estimate the proportion of distinct viral lineages and the proportion of anti-viral drug resistance mutations (and neutralization mutations for HBV) in incident and prevalent blood donor infections. We estimate, based on previous observations, that approximately 40-50 incident HIV infections, 25-50 incident HCV infections, and at least 50 incident HBV infections will be identified per year, while an equal number of prevalent infections will be tested per year. Therefore, over the course of this 3-year study, we anticipate conducting genotypic testing on 120-150 incident and 120-150 prevalent HIV infections, 75-150 incident and 75-150 prevalent HCV infections, and 150

incident and 150 prevalent HBV infections. Table F.1.1 shows the precision we may expect considering various plausible genotypic frequencies and assuming that genotypic information is available on 50 HIV, 50 HCV and 50 HBV incident infections per year.

For example, if 150 HIV incident infections are genotyped over the 3-year study period and 3 are found to be of a non-B subtype (observed proportion of 2.0%), then the 95% confidence interval (CI) for the proportion of non-B subtypes among HIV incident donors will be 0.4% to 5.7%. Similarly, the 95% CI around the proportion of HIV drug-resistance mutations if the latter was observed to be 10.0%, would be 5.7% to 16.0%.

Table F.1.1. 95% confidence interval for given frequencies and sample sizes.

					95% Confidence Interval	
Sample size	**Virus**	**Subgroup**	**plausible observed frequency**	**proportion**	**Lower limit**	**Upper limit**
150	**HIV**	**Non-B subtype**	3	2.0%	0.4%	5.7%
		Mutation	15	10.0%	5.7%	16.0%
50		**Non-B subtype**	1	2.0%	0.1%	10.6%
		Mutation	5	10.0%	3.3%	21.8%
150	**HCV**	**1a**	105	70.0%	62.0%	77.2%
		1b	30	20.0%	13.9%	27.3%
		2b	8	5.3%	2.3%	10.2%
		3a	5	3.3%	1.1%	7.6%
50		**1a**	35	70.0%	55.4%	82.1%
		1b	10	20.0%	10.0%	33.7%
		2b	3	6.0%	1.3%	16.5%
		3a	2	4.0%	0.5%	13.7%
150	**HBV**	**A**	57	38.0%	30.2%	46.3%
		B	33	22.0%	15.7%	29.5%
		C	45	30.0%	22.8%	38.0%
		D	15	10.0%	5.7%	16.0%
		E	1	0.7%	0.0%	3.7%
		F	1	0.7%	0.0%	3.7%
		G	2	1.3%	0.2%	4.7%
		neutralization	10	6.7%	3.2%	11.9%
		Drug resistance	14	9.3%	5.2%	15.2%
50		**A**	19	38.0%	24.7%	52.8%
		B	11	22.0%	11.5%	36.0%
		C	15	30.0%	17.9%	44.6%
		D	5	10.0%	3.3%	21.8%
		E	0	0.0%	0.0%	7.1%
		F	0	0.0%	0.0%	7.1%
		G	1	2.0%	0.1%	10.6%
		neutralization	3	6.0%	1.3%	16.5%
		Drug resistance	5	10.0%	3.3%	21.8%

F.2. Power Calculations:

This study's primary objective is to provide a description of the distinct viral lineages and evaluate the frequency of anti-viral drug resistance mutations in blood donors. We will also explore if genotypic distributions and mutation frequencies appear to differ between groups recognizing that frequencies will

not be found to be statistically significantly different from one another unless differences in frequencies between groups are large. Analyses for these secondary objectives are exploratory in nature and the study is not powered around these objectives. Thus, as examples, we only provide below power calculations for two of the secondary objectives to illustrate the magnitude of the differences that would be detectable with 80% power in this study.

One of the secondary objective in this study is to explore the hypothesis that the frequencies of different lineages and/or drug and neutralization resistant variants are different between incident and prevalent cases. If we assume that the frequency of HIV non-B subtype in prevalent infections is 2% and if we genotype 150 incident cases and 150 prevalent HIV infections, we will have 80% power to detect a significant difference (alpha=0.05) in the frequencies of non-B subtype between HIV incident and prevalent infections if the frequency of HIV non-B subtype is 10.0% among HIV incident cases (Table F.2.1). Likewise, if 10% of prevalent infections have drug resistance mutations, we will have 80% power to detect a significant difference in the frequencies of drug-resistance mutations if the frequency is either 22.4% or 2.0% among HIV incident cases (Table F.2.1). Table F.2.1 shows similar calculations for HBV and HCV genotypes and mutations.

Table F.2.1. Viral genotypic and mutation frequencies in prevalent and incident infections that would be found to be statistically significantly different from one another (alpha=0.05, power=80%)

				80% Power	
			Hypothesized Frequency in Prevalent cases	Hypothesized Frequency in Incident cases for a significant difference to be detected	
virus	prevalent and incident sample sizes	subgroup		Lower frequency	Higher frequency
HIV	150	non-B subtype	2.0%	–	10.0%
		mutation	10.0%	2.0%	22.4%
HCV	150	1a	69.8%	53.5%	83.9%
		1b	20.6%	8.7%	35.6%
		2b	5.1%	–	15.2%
		3a	3.3%	–	12.3%
HBV	150	A	37.4%	22.1%	53.9%
		B	22.0%	9.7%	37.3%
		C	30.8%	16.5%	47.1%
		D	10.4%	2.2%	23.0%
		E	0.4%	–	6.3%
		F	0.6%	–	6.9%
		G	1.1%	–	8.1%
		neutralization	6.6%	0.5%	17.5%
		drug resistance	9.2%	1.6%	21.3%

We will also examine the hypothesis that the frequencies of different lineages and drug/neutralization resistant variants in incident cases vary significantly over time (2006-2008). To examine this hypothesis, we are assuming that there will be approximately 50 HIV, 50 HBV, and 50 HCV incident cases identified per year. As shown in Table F.2.2, under the assumption that 2% of incident HIV cases are non-B subtype in year 1 of this study (2006), we will have 80% power (with an alpha of 0.05) to detect an annual increase of 8.0% per year (i.e. 10.0 % in year 2 and 18.0% in year 3). Likewise, Table F.2.2 shows the annual % increase or decrease that could be statistically detected for each of the viral lineages and drug/neutralization resistant variants.

Table F.2.2. Temporal changes in frequencies that would be detected with 80% power and alpha of 0.05.

				80% Power	
				hypothesized change in frequency per year	
Virus	**incident sample size per year**	**subgroup**	**hypothesized frequency in first year**	**frequency decrease per year**	**frequency increase per year**
HIV	**50**	**non-B subtype**	2.0%	–	8.0%/yr
		mutation	10.0%	–	11.2%/yr
HCV	**50**	**1a**	69.8%	13.8%/yr	10.8%/yr
		1b	20.6%	8.7%/yr	13.1%/yr
		2b	5.1%	–	9.6%/yr
		3a	3.3%	–	8.8%/yr
HBV	**50**	**A**	37.4%	12.0%/yr	13.8%/yr
		B	22.0%	9.1%/yr	13.2%/yr
		C	30.8%	10.9%/yr	13.8%/yr
		D	10.4%	–	12.3%/yr
		E	0.4%	–	6.7%/yr
		F	0.6%	–	6.9%/yr
		G	1.1%	–	7.3%/yr
		neutralization	6.6%	–	10.2%/yr
		Drug resistance	9.2%	–	11.0%/yr

G. Data Compilation for Analysis

Westat will merge all genotypic and mutation test result information provided by BSRI, original viral confirmatory test results, and demographic information. When linking these data to form the analytical dataset, Westat will automatically transform donations' "3-digit zip codes" to "CDC geographical region codes", "birth dates" to "age (in years) at donation", and "donation date" to "year of donation". The resulting dataset will thus only include BUIs, viral test results, and general demographic information (the latter cannot permit identification of a donor). Confidentiality of data will thus be maximized since Westat (or BSRI) never receives the link between BUIs and donor identifying information (this link is maintained at each blood center and never provided to Westat or BSRI). This merge will probably be conducted every 6 months to facilitate the QC process and allow for the rapid formation of a final dataset after all laboratory testing is completed. The resulting 2006-2008 SAS dataset will be used to conduct all analyses as detailed in Section H below.

The summary statistic files will be compiled to allow for formation of a summary dataset that includes for each year, the total number of donations screened and their breakdown by first-time/repeat status, age and gender (information on race/ethnicity and geographical area may also be available). This summary dataset will allow us to describe the population screened and to calculate overall rates, as warranted.

H. Data Analysis

We will first calculate the proportion (frequency) and associated 95% CI of each viral lineage and drug resistant/neutralization variant based on the laboratory results separately for prevalent and incident infections. To evaluate if the proportions of different lineages and/or drug and neutralization resistant variants vary by year, by demographics, or between incident and prevalent cases, we will produce frequency tables and use Fisher's Exact Tests. Further, exploratory logistic regression models may be conducted with viral lineage or drug resistance/neutralization variants as outcomes and type of infection

(incident/prevalent), year, and demographics as independent variables. For example, the logit probability of an HIV case being non-B subtype can be modeled as a function of whether the HIV case was determined to be an incident or prevalent case, the year the case was identified, the age of the donor, the gender of the donor, and other demographics and risk factors pertaining to the donor. These models would allow us to estimate whether the odds of having a particular genotypic variant differs between incident and prevalent cases or by demographics while adjusting for other variables. SAS will be used for all analyses.

I. Human subject considerations

Prior to donation, blood donors routinely sign a consent form agreeing that their blood samples will be tested for infectious diseases such as HIV, HBV, and HCV. Additionally, at many blood centers, donors sign a consent form agreeing that their blood samples may be used for research purposes. Blood donations have been used for similar research studies in the recent past such as subtyping HIV in US blood donors (Delwart et al., 2003). Requesting that all donors sign a separate consent form prior to blood donations for the purpose of this study is therefore not warranted.

Between 2006 and 2008, samples will be collected from selected HIV, HCV and HBV positive blood donors by Blood Systems, American Red Cross, New York Blood Center and Retrovirus Epidemiology Donor Study-II (REDS-II) centers which are not part of Blood Systems or the American Red Cross (Blood Center of Wisconsin, Hoxworth Blood Center-Cincinnati OH, Institute for Transfusion Medicine, Pittsburgh PA). Samples received by BSRI for viral genetic analyses will be identified by blood unit numbers (BUIs) and virus detected (plus center if this information is not included in the BUI). Only blood collection centers will have the link between the BUI and the personal identifiers such as name and addresses, and this link will never be shared with BSRI or Westat. Further, the analytical dataset will only include for each BUI, information on CDC geographical region code, year of collection, race/ethnicity (if available), gender, and age (in years) ensuring that donors could not be identified from the demographic information present on the analytical dataset. Approval from all appropriate IRBs will be sought before any shipment or testing of samples occurs and before any data file is sent to BSRI or Westat.

Donors in whom evidence of HIV, HCV or HBV infection(s) are detected are now routinely contacted by blood collecting centers. At that time, they are notified with regards to the health implications of their results and ways to reduce viral transmission. In this study, blood centers will also notify donors who are found to have HIV drug resistance mutations since this may impact their treatment strategies. However, blood donors will not be notified of results from other analyses in this study.

Prior studies have indicated that approximately 10% of recently infected subjects harbor HIV with significant drug resistance mutations (Little et al, 2002, Weinstock et al, 2004). HIV drug resistance genotyping can now be performed commercially. Many doctors do not acquire any drug resistance mutation data on their infected patients prior to initiating anti-viral treatment and simply monitor patients' viral load to determine if the therapy is effective. Other people initiating anti-HIV therapies are counseled by their health care providers to determine their drug resistance viral genotypes immediately prior to starting treatment to provide a better estimate of their likely response to anti-viral therapy. Because of the potential clinical impact of drug resistance mutations on response to anti-retroviral therapy, infected donors in which drug resistance mutations are found in both protease and reverse transcriptase genes (http://www.iasusa.org/resistance_mutations/index.html) will be contacted and informed of their drug resistance mutation and its potential impact on anti-retroviral therapy responses. Because the drug resistance data will be generated 6 to 12 months after their blood donation, the ability to contact such donors will be limited but best faith efforts will be made to inform donors carrying HIV with evidence of drug resistance mutations. Notification of donors with drug resistant HIV mutations will be

performed by the medical director of the blood center where the donor gave blood and was identified as having a positive HIV test.

Because of the impact of HCV genotype on response to treatment modalities, patients considering anti-HCV treatment have, as part of standard medical practice, their virus commercially genotyped prior to initiating treatment. The results from this study will therefore be duplicating HCV genotyping results for donors already considering treatment. Therefore, donors will not be notified of these genotyping results.

HBV genotype is not known to impact treatment outcome and HBV neutralization sensitivity is of no consequence to a person already chronically infected with HBV as it is only used for prophylactic purposes. In fact, the standard of care for HBV does not include obtaining a drug resistance genotype prior to therapy. HBV drug resistance genotyping is therefore used only for patients failing their therapy after long term anti-viral treatments. Donors will therefore not be notified of these genotyping.

Because of the reasons mentioned above, the donors will therefore not be notified of the result of the genetic analyses of their viruses unless HIV drug resistance is detected.

J. Timeline

In the beginning of 2006, the BSRI lab will order PCR primers for HIV, HCV and HBV and optimize their application using available samples. After all IRB approvals are received, we anticipate that HCV and HBV prevalent infection samples will be received in September 2006 so they can start to be processed. A shipment of incident infection samples and of HIV-antibody positive samples will be received every 5-6 months beginning in September 2006, processed and sequenced. Table J below shows the proposed schedule for the study:

Table J.

February 2006	Steering Committee
February 2006	Executive Committee
June-August 2006	OSMB
June-August 2006	IRB review (ARC, UBS/BCP/UCSF, Westat, Hoxworth, ITxM, BCW, NYBC); Optimization of PCR protocols for HIV, HCV, HBV
September 2006	HCV and HBV Prevalent case samples shipped to BSRI; List with demographics sent to Westat
September 2006	Batch 1 of incident and HIV Ab-positive case samples shipped to BSRI; List with demographics sent to Westat
September – February 2007	Testing at BSRI
February 2007	Testing information for batch 1 sent to Westat; Westat compiles testing results into database with demographic information
March 2007	Batch 2 of incident and HIV Ab-positive case samples shipped to BSRI; List with demographics sent to Westat
March 2007	Summary statistic data for 2006 sent to Westat
March – August 2007	Testing at BSRI
August 2007	Testing information for batch 2 sent to Westat; Westat compiles testing results into database with demographic information
September 2007	Batch 3 of incident and HIV Ab-positive case samples shipped to BSRI; List with demographics sent to Westat
September – January 2008	Testing at BSRI
January 2008	Testing information for batch 3 sent to Westat; Westat compiles testing results into database with demographic information
February 2008	Batch 4 of incident and HIV Ab-positive case samples shipped to BSRI; List with demographics sent to Westat
March 2008	Summary statistic data for 2007 sent to Westat
February – June 2008	Testing at BSRI
June 2008	Testing information for batch 4 sent to Westat; Westat compiles testing results into database with demographic information
July 2008	Batch 5 of incident and HIV Ab-positive case samples shipped to BSRI; List with demographics sent to Westat
July – December 2008	Testing at BSRI
December 2008	Testing information for batch 5 sent to Westat; Westat compiles testing results into database with demographic information
January 2009	Batch 6 of incident and HIV Ab+ case samples shipped to BSRI; List with demographics sent to Westat; Summary statistic data sent to Westat
January – May 2009	Testing at BSRI
March 2009	Summary statistic data for 2008 sent to Westat
May 2009	Testing information for batch 6 sent to Westat;
May-June 2009	Westat compiles testing results into database with demographic information and produces a cumulative SAS dataset
June-August 2009	Data analysis and interpretation

K. Budget

	Direct and Indirect Costs
Blood Centers and Laboratories	$ 411,847
REDS-II Central Laboratory	$574,127
REDS-II Coordinating Center	$400,197
Total	$1,386,170

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Appendix I.

Estimates of the number of allogeneic donations, number of incident HIV, HCV, and HBV infections, and number of first-time HIV, HCV, and HBV prevalent infections per year

	Allogeneic Donations		HIV-1 Incident	HIV-1 Prevalent	HCV Incident	HCV Prevalent	HBV Incident	HBV Prevalent
	First-time	Repeat	HIV-NATpos HIV-Ab neg or recent† HIV-NAT pos HIV-Ab pos	First-time Remote† HIV-NAT pos HIV-Ab pos	HCV-NAT pos HCV-Ab neg	First-time HCV-NAT pos HCV-Ab pos	Anti-HBc neg HBsAg pos	First-time Anti-HBc pos HBsAg pos
	n	n	n	n	n	n	n	n
REDS-II Centers and NYBC	141,820	728,200	12	20	5	218	31	135
UBS	137,754	618,536	7	24	3	389	26	86
ARC	1,151,582	5,403,506	39	90	26	1,645	99	715
Projected Total[ʃ]	*1,431,156*	*6,750,242*	*58*	*134*	*34*	*2,252*	*156*	*936*

*Allogeneic donations only include whole blood community, directed, and apheresis donations.

† : 25% of all allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be recent infections (within the last 6 months) while 75% of all allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be remote infections (more than 6 months ago).

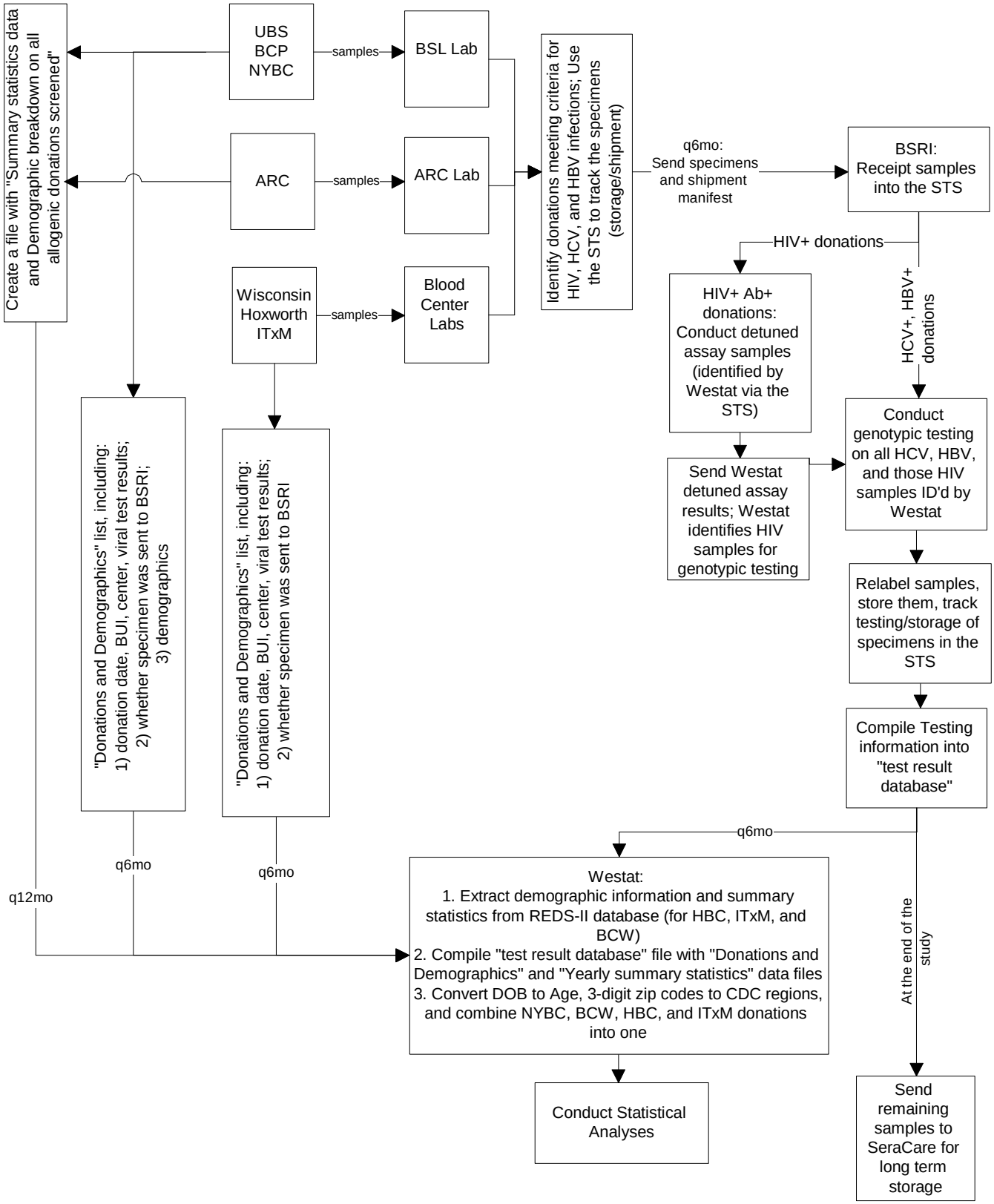
10% of first time allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be recent infections (less than 6 months ago) while 90% of first-time allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be remote infections (more than 6 months ago).

50% of repeat allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be recent infections (less than 6 months ago) while 50% of repeat allogeneic donations that are HIV-NAT positive and HIV-antibody positive are assumed to be remote infections (more than 6 months ago).

ʃ: The total number of infections were projected based on yield of cases in 2004.

Appendix II.

Flow chart of Study Design



Demographic data = Age; 3-digit zip code; race/ethnicity (if available); gender; first time/repeat donor status