RFS# 005

In response to: Reproductive Success of Natural-Origin, Hatchery-Origin, and Reconditioned Kelt Steelhead (FCRPS BIOp Action #184)

Assessment of the Reproductive Success of Reconditioned Kelt Steelhead with DNA Microarray Technology

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Summary.

Need: The Federal Colombia River Power System (FCRPS) has developed a comprehensive Research, Monitoring and Evaluation program (RME) to track the progress of mitigation measures listed in the National Oceanographic and Atmospheric Administration Fisheries' (NOAA) Biological Opinion (BiOp). A central feature of the BiOp is to implement systems and measures that provide biological benefit to endangered or threatened fish stocks in the Columbia river basin. In particular, the RME Hatchery/Harvest subgroup identified the critical need "to synthesize an analytical approach to determine the effects of hatchery reforms on extinction risk and recovery ... of steelhead populations in the Columbia river basin (RPA Action 184)".

In response to Action 184, the Bonneville Power Administration seeks research proposals addressing the reproductive success of natural-origin, hatchery-origin and reconditioned kelt steelhead. *Critical to the success of the RME in general and RPA Action 184 in particular is a capability to accurately identify and categorize individual fish with their origin or stock, and fish progeny with their parents.*

The DNA of each fish is unique, and therefore serves as a unique tag, or fingerprint, for that animal. Further, DNA provides unequivical information about a fish's pedigree. Whereas traditional microsatellite/DNA fingerprinting techniques based on PCR and gel electrophoresis technologies can address many paternity and species identification questions, even high-resolution sequencing gels lack the necessary resolution, reproducibility and throughput to identify DNA signatures related to hatchery/wild origins and fitness. DNA microarray technology solves the inherent resolution and reproducibility issues associated with gel technology by fixing (up to 10^6) discreet DNA signatures in a (1 x 1 cm^2) physical space, and identifying microsatellites or other DNA signatures based on their nucleotide sequence rather than fragment size. Methods for quantitative data extraction and analysis from microarrays have already been developed, thereby providing a statistical estimate of uncertainty for every "band" in every "fingerprint" and for every biological conclusion. For these reasons, microarray technology has the potential to generate a unique and biologically informative fingerprint for establishing the pedigree and geographical, drainage, or stock origin of each sampled fish. Hence, DNA microarray technology provides a capability critical to the success of the RME.

Objectives: DNA microarray technology potentially provides BPA with *the capability to accurately identify fish with their stock, and fish progeny with their parents.* To realize this potential, this study will use Argonne (ANL) and Pacific Northwest National Laboratories' (BATTELLE) existing DNA microarray technology, BATTELLE's statistics resources and samples from the ongoing Yakima Nation kelt reconditioning project (BPA Project 200001700) to:

1. link steelhead progeny in the F2 generation to their parents in the F1generation comprised of both first-time spawners and reconditioned kelts from the same geographic stock;
2. estimate reproductive success of first-time spawners and reconditioned kelts; and

3. adapt and then make DNA microarray fingerprinting methodology accessible to other Action 182 and 184 problems.

Milestones/Deliverables:
Year 1: steelhead-focused microarray design; preliminary steelhead-focused microarray data extraction, management and analysis software; preliminary standard operating protocol; preliminary assessment of the reproductive success of reconditioned kelts.
Year 2: refined data extraction, management and analysis software; refined standard operating protocol; preliminary steelhead DNA microarray fingerprint library; refined assessment of reproductive success of reconditioned kelts.
Year 3: extended DNA microarray fingerprint library for select steelhead stocks; open-source library software and protocols for fingerprint referencing, querying and summarization; comparative study of microarray and gel-based fingerprinting technologies in collaboration with CRITFC researchers.
Year 4: extended DNA fingerprint library including other stocks sampled under BPA project 200001700; fingerprint library, microarray technology and methodology transfer to CRITFC or other suitable group.

Impact: The steelhead DNA-fingerprinting microarray takes advantage of salmonid genome structure, organization, DNA sequence and single nucleotide mismatch discrimination, all without *a priori* knowledge of an organisms' DNA sequence or structure. Whereas DNA microsatellite gel techniques take advantage of microsatelite size, the DNA microarray takes advantage of microsatelite sequence. This research results in a methodology to interrogate and identify steelhead with their stock, parents and progeny. This methodology provides a fundamental and critical capability for the successful execution of the FCRPS RME.

Estimated Cost: $462k/year over 4 years or $1.848 millon total.

Description.

Our goal is to adapt DNA microarray fingerprinting technology and statistical sampling, estimation and analysis methods to link steelhead progeny in the F2 generation to their parents in the F1generation, where the F1 generation is comprised of both first-time spawners and hatchery reconditioned kelts from the same geographic stock. The ability to unambiguously assign parentage to the F2 offspring would provide a robust means of assessing the relative reproductive success rates of reconditioned steelhead kelts and first-time spawners from the same stock. This information could be used to directly address the uncertainties surrounding assessment of the potential benefits of actively promoting iteroparity as a strategy for rebuilding depressed ESU steelhead populations.

Central to the question of reproductive fitness and iteroparity are the genetic definitions and distinctions of individual steelhead and/or stocks. Current genetic statements about salmonids ultimately constitute genetic hypotheses, not statements of fact. Unfortunately, these hypotheses have gone largely untested due to inadequate statistical resolution afforded by gel-based fingerprinting techniques, and the high cost and time commitments

necessary to generate and interpret the data. Thus, techniques using genetic information to distinguish between salmonid populations remain largely under-developed. Addressing hypotheses regarding differential reproductive success between genetically distinct salmonid populations first requires a robust, statistically-based and defensible means of genetically differentiating between individuals in a population. DNA microarrays are relatively rapid and inexpensive data generating tools that provide a technological solution to these problems, because they provide a high-throughput, robust platform for assessing the statistical confidence and accuracy of deduced genetic relationships between individuals within and between populations.

Research conducted at Pacific Northwest National Laboratory in FY01 and FY02 demonstrated that DNA microarray fingerprinting and associated statistical technology allow reliable differentiation of salmonid stocks. Preliminary results from this research also showed reliable differentiation between individuals within certain stocks.

The scope of this proposal involves fingerprinting individuals over four years from Yakima River steelhead stocks that are listed as part of the Mid-Columbia River steelhead ESU and possibly extending fingerprinting in years 3 and 4 to include steelhead enhancement programs being conducted on the Okanogan, Umatilla, Imnaha and Grande Ronde rivers. Dr. David Fast of the Yakima Nations Fisheries Resource Management (YNFRM) group, under the Yakima Klickitat Fisheries Project (YKFP), currently leads on-going studies[1] of the Yakima River steelhead stocks. The genetic microarray studies proposed here will compliment Dr. Fast's current work, and aid in addressing some of the specific goals of the YKFP as well as the efficacy of kelt reconditioning in the Yakima basin drainages. Tissue samples for genetic microarray analysis will be obtained through cooperative efforts with the YKFP projects. Genetic characterization and discrimination methods (already developed and applied to salmonids at BATTELLE) will be applied on a limited and known number of distinct steelhead stocks comprised of known reconditioned and released kelts and first-time steelhead spawners from a sub-basin of the Yakima River drainage.

These genetic characterization tools will be applied to the known population of F1 steelhead kelts and first-time spawners for each drainage included in the YKFP kelt reconditioning study. Upstream and downstream passage of adult steelhead will be controlled by weirs located below the main spawning areas of these drainages. Initially, F2 progeny (fry and par) will be collected, tissue sampled, and released from the reaches above the weirs where the candidate parents were known to have gone. These F2 samples will be genetically matched back to the candidate parental stock and provide a first level of assessment on reproductive success between kelts and first-time spawners. Subsequent tissue sampling and genetic characterization of upstream migrating adults past these weirs will provide a means to assess the smolt-to-adult reproductive success rates.

It is highly anticipated that validation and refinement of methods realized in this study will be immediately extensible to other ESU steelhead stocks within the Columbia River

[1] Kelt Reconditioning: A Research Project to Enhance Iteroparity in Columbia Basin Steelhead (Oncorhynchus mykiss). BPA project 200001700.

and Snake River Basins. It is further anticipated that DNA microarray technologies could be applied to ESU salmon populations within these same basins and provide a new and effective tool for fishery managers to address a variety of problems in salmon fisheries management in these and other areas.

Concerns related to the genetic integrity of Columbia Basin fish are stated throughout the reasonable and prudent alternatives section of the 2000 FCRPS Biological Opinion (2). The authors of "Upstream: Salmon and Society in the Pacific Northwest" write: "Managing salmon requires an understanding of the biologic al dynamics of the populations in which they occur and reproduce. In particular, knowledge of the structure of the genetic variation in salmon is needed to make decisions about how to identify and protect the local reproductive units, which are the fundamental biological units" (NRC 1995). And, "The overarching goal of Columbia Basin Fish and Wildlife Authority (CBFWA) is to restore sustainable, natural-producing fish…populations…by restoring the biological integrity and the genetic diversity of the Columbia River ecosystem" (Draft FY 1998 AIWP, Appendix A, 6/4/97).

Key factors for recovery and protection are the extent to which the impacts on salmon populations affect the genetic integrity of Columbia Basin salmonids, and the extent to which that genetic information can be used to inform management policy and implementation. The genetic information needed to make informed decisions on each of these factors cannot currently be obtained accurately, inexpensively, and on a timely basis using existing techniques and data (described below). Overriding these specific concerns (timeliness, accuracy, and cost) is the need to enhance the interaction of genetics and fishery management, which will only be accomplished with the development of genetic and statistical tools that resolve these concerns (44).

State-of-the-Science. The value of genetic techniques to address fundamental fisheries management questions is well recognized (5, 6, 11, 12, 16-18, 21, 22, 28, 31, 32, 34, 36-38, 41, 48, 49). Current investigations of fish stocks and population structure utilize fairly standard techniques for isozyme analysis and/or DNA fingerprinting. These include starch gel electrophoresis, immuno-histochemical staining, variations on southern hybridization (52, 55) or polymerase chain reaction (PCR)-based techniques such as randomly amplified polymorphic DNA (RAPD) typing (7, 54), restriction fragment length polymorphism (RFLP) of nuclear or mitochondrial DNA (mtDNA), or combinations thereof. Hypervariable mini- (9-100 bp) and micro- (2-6 bp) satellites and variable number of tandem repeat (VNTR) loci are becoming even more popular for fisheries management; because they occur frequently and are uniformly distributed throughout the genome, they are highly variable and individual-specific (reviewed by

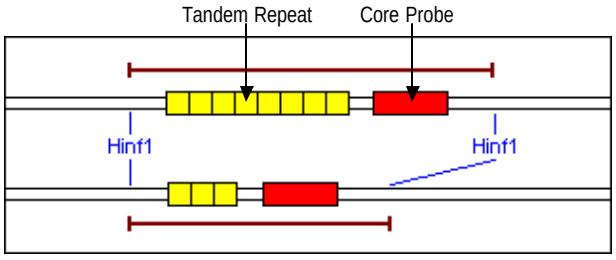

Figure 1. Representation of a VNTR locus detected by Southern blot. Each allele contains a different number of tandem repeat sequence, which are resolved as different DNA fragment sizes on an agarose or polyacrylamide gel when genomic DNA is digested with the enzyme Hinf1 and detected with the core probe. The alleles can also be visualized by PCR amplification with conserved primers flanking the VNTR region.

O'Reilley and Wright (37) and Ferguson et al. (14)). Standard methods either analyze a single locus at a time (e.g., PCR-based typing), or multiple loci simultaneously (e.g., Southern blot methods). Invariably, these fingerprinting techniques are predicated upon resolving differences in repeat (e.g., $(CA)_n$) numbers (Figure 1) and accurately determining (by gel electrophoresis) differences in fragment *length.*

Limitations to Gel Electrophoresis for DNAFingerprinting. Some of the strengths and weaknesses of current fingerprinting techniques are reviewed in (14). Ultimately, it is not DNA technology or DNA fingerprinting *per se* that precludes managers from making timely, informed decisions on hatchery operations for the benefit of weak stocks. Rather, there are technological limitations in both the *PCR* and *gel-based sizing methods* in common use. Given the multilocus tag shown in Figure 2, for example, we can easily discern some of the limitations and tenuous assumptions of gel-based techniques:

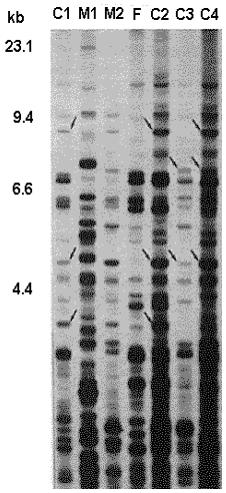

Figure 2. A hypothetical multilocus DNA fingerprint resolved by agarose gel electrophoresis. Fragment sizes (in kilobases) are indicated on the left.

1. *What constitutes a band?* Not all amplification products or restriction fragments are readily resolved by either agarose *or* polyacrylamide gels. What is the *statistical* definition of a band? What are the statistical criteria for separating or combining bands into unique bins? Are "wide" bands really singlets, doublets, or triplets? Microarrays interrogate DNA fragments at the nucleotide *sequence* level, providing unambiguous identification to those fragments containing a core probe, mini- or micro-satellite.

2. *What are the statistical criteria for including or excluding data?* Bands below 2 Kb or above 10Kb are frequently discarded from the statistical analysis. How does background hybridization and smearing affect the quality of the data or analysis? All of the data from a microarray can be included in the analysis, providing more loci, replication, and confidence in the resulting tag or genetic conclusion.

3. *How are tags compared across gels, over time, and between laboratories?* Gels are not static or invariant. Electrophoresis, buffer anomalies, air bubbles and temperature effects introduce smiles, bends, warps or other band shifts/distortions. Even with advanced image analysis software, rectifying these anomalies requires a subjective decision regarding band identities and similarities across lanes. Statistical tools for confidently comparing tags from many gels, acquired at different times or locations do not exist. The net result of this limitation is tremendous uncertainty in the resulting genetic data, which may result in a different management option or hatchery operation than would otherwise occur with more robust and statistically rigorous (raw) data.

4. *Do band intensities contain useful, discriminatory information?* Band intensities are not currently factored into standard DNA fingerprinting analyses.

5. *PCR is not perfect*. Amplification through VNTR loci is subject to PCR stutter, slippage and chimera formation, all of which lead to factually incorrect genetic data and tags. Additional, practical limitations to existing DNA fingerprinting technology are the time, expense and technical expertise required to perform the analysis.

6. *Limited replication*. In all cases, gel-based analytical methods preclude sufficient replication (fingerprints per fish and number of fish fingerprinted) to provide robust, reproducible, defensible data.

7. *Standardization*. There are literally thousands of mini- and microsatellites within the salmonid genome, with no accepted standard of genetic comparison from one study to the next. Microarray technology gives geneticists the option to analyze literally thousands of loci simultaneously, providing a standardized genetic tool for all subsequent studies.

8. *Time to result*. Gel-based fingerprinting data requires months to analyze and comprehend, precluding timely and meaningful alterations in fisheries operations for the benefit of weak stocks. DNA microarray technology can provide a genetic tag within 24 hours (or sooner), which provides managers the option to alter fisheries operations in near-real time.

9. *Cost*. Gel-based techniques (including those based on ABI 377 sequencers) are technically demanding, require significant manual intervention, and utilize fairly expensive reagents. An analytical method based on DNA microarrays, on the other hand, can be fully automated (from the point of DNA extraction through data analysis) and costs no more than a coded wire tag.

10. *Information generation.* Gels can generate 10 to 20 bits of information, compared to hundreds or thousands of bits of information from a single DNA microarray. The cost/bit and time/bit are lower for microarrays by several orders of magnitude; hence, the practical resolving power of microarrays cannot be approached by gel technology.

DNA Microarray Technology: From Fragment Sizing to Fragment Sequencing. We contend that applying DNA microarrays to traditional areas of genetic stock identification will provide a significant technological solution to problems related to the aforementioned limitations in gel-based genetic techniques, especially relative to reducing uncertainty and providing managers with timely genetic information that can be incorporated into fisheries management decisions. The automated technology and statistical algorithms developed at Argonne National and Pacific Northwest National Laboratories (ANL & BATTELLE) will generate more information, more quickly and at less cost than traditional methods, while resolving the uncertainties that complicate the use of DNA information for managing fish populations.

Microarrays typically contain hundreds, thousands, or hundreds of thousands of individual nucleic acid probes addressed at specific locations within a 1 x 1 cm chip, and were originally developed for large-scale DNA sequencing projects, clinical diagnostics

and genetic analyses (8, 10, 39, 42, 46, 50, 56). Thus, a single microarray can accommodate all of the necessary probes required for statistically rigorous individual or stock identification. Further, the microarray accesses information and interrogates the genome directly at the sequence level instead of relying solely on post-PCR size discrimination of resulting DNA fragments (as with gel-based detection systems) or limited sequence sampling (e.g. restriction enzyme analysis). Since many hundreds of probe sequences can be arrayed in a very small area (e.g. a micro-titer plate well), numerous tissues or independent fish samples can be analyzed simultaneously with existing robotic systems, minimal manual intervention and at minimal cost. Single

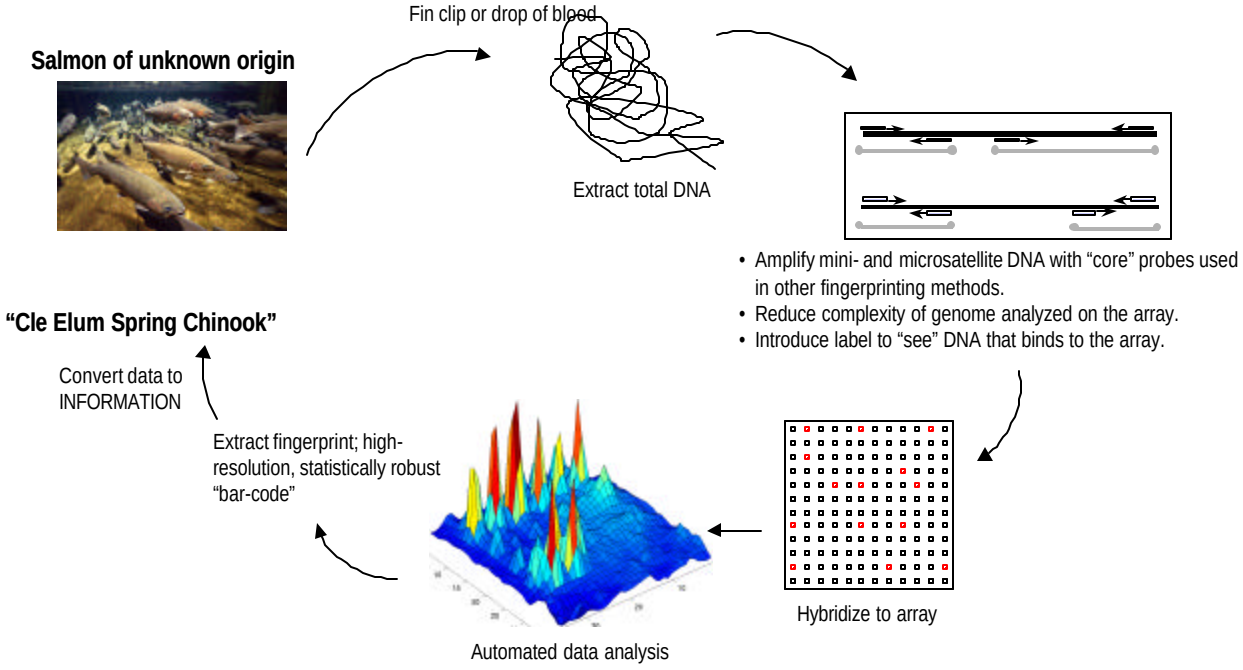

Figure 3. The microarray fingerprinting method.

nucleotide mismatches are also easily discriminated by microarray hybridization (13, 19, 20, 30, 39, 40). Therefore, DNA microarrays offer tremendous potential for stock identification and characterization in both basic and applied natural resource science, overcoming many of the technological, practical and cost limitations of current fingerprinting technologies.

Through a 2-year BATTELLE-supported project, we have developed a prototype fish fingerprinting microarray, statistical algorithms and protocols. We have applied these to the genetic identification of salmonids. Details of our methods and preliminary results with salmonid fingerprinting are described below.

Preliminary Studies. A DNA microarray is conceptually analogous to the more traditional fingerprinting methods. Thus, we are not proposing a new technology that requires the displacement of information learned from past technology. Instead, we are bringing new technology to a region that will ultimately make DNA information more useful to fish managers. PCR primers and genomic DNA targets are roughly identical to specific loci within the genome, while the microarray probes identify specific alleles or

forms of those loci. Target loci follow all of the rules and assumptions established for VNTRs and microsatellites (e.g. selectively neutral, independent segregation, etc.), such that the microarray method is applicable to the same situations and biological questions where gel-based DNA fingerprinting has already been applied with great success.

The analytical process of the universal fingerprinting chip is depicted in Figure 3. Genomic DNA is extracted from small fin clips, blood or tissue samples collected according to standard techniques. These genetic samples are amplified with repetitive DNA PCR primers targeting those genetic elements used in current gel-based fingerprinting approaches. Rather than resolve fragments on a gel, however, we hybridize the amplified fragments to an oligonucleotide microarray containing immobilized 9- or 10-base (or 10-mer) capture probes as illustrated in Figure 4. Converting a microarray image into a meaningful, statistically robust tag or fingerprint is non-trivial, and continues to be a significant limitation to many microarray experiments and traditional fingerprinting applications. It is precisely because we have addressed some of the statistical and image analysis issues surrounding microarray technology that we can extract a quantitative microarray tag from a fish, and quantitatively compare it to microarray tags of other fish. Here, we detail our technical approach, and identify the next steps in the technology that constitute the technical objectives of this proposal.

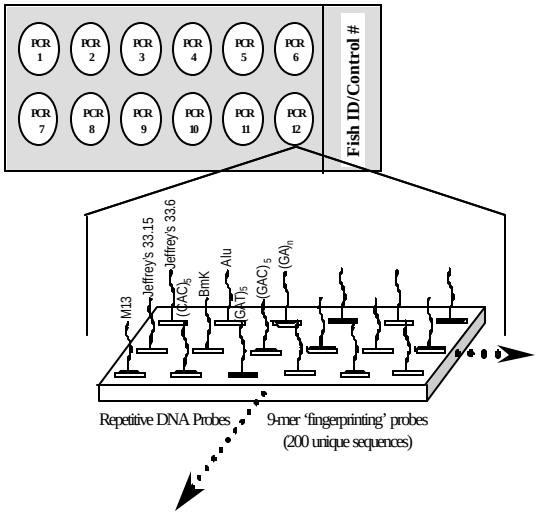

Figure 4. Conceptual representation of the DNA fingerprinting chip. Each 1 x 3 inch glass slide contains 12 independent hybridization wells that are defined by a teflon mask (gray). Each well is/can be hybridized with a different pool of PCR products arising from repetitive DNA-PCR amplification of genomic DNA. Each well contains an independent array of identical fingerprinting probes and an appropriate complement of control probes/spots. The diagnostic portion of the prototype array consists of 200 unique 9-mer fingerprinting probes.

Genome Sampling: Caudal fin clips were obtained from adult Priest Rapids fall chinook. High molecular weight chromosomal DNA was isolated from fin clips using Qiagen DNeasy Tissue kits according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). For our preliminary studies, we used Jeffreys' 33.15 repetitive DNA probe (25) and the salmonid-specific OMY77 repetitive element (31) as PCR primers to sample the salmon genomes and generate amplified genomic DNA fragments for subsequent analysis on the random oligonucleotide microarray. At least two replicate PCR amplifications were performed for every fish and test condition; indeed, replication of the entire analytical process was key to our statistical methods for estimating a microbial tag (described below). PCR amplifications were performed in 50 µL total volume, using an MJ Research Tetrad Thermal cycler and 96 well plates (MJ Research, Watertown, MA), 100 ng genomic DNA and: 1X PCR buffer (Qiagen), 3.5 mM Mg^{2+}, 200 µM each dNTP, 1U *Taq* polymerase, and 0.2 µM of the fluorescently-labeled PCR primer. Thermal cycling conditions were 95°C for 15 minutes followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 3 min, and cooling to 4°C. PCR amplification was confirmed by analyzing 20 µL aliquots of the amplification reaction on 2% agarose gels in 1X TAE

running buffer prior to microarray analysis. The remaining, labeled amplification products were hybridized directly to microarrays without further manipulation, as described below. Post-PCR cleanup of amplification products did not alter the tag profiles (not shown), and was therefore unnecessary.

Microarray Probes: The original prototype fingerprinting microarray was custom-designed and manufactured in our laboratory. A list of 2000 nonamer microarray capture probes was generated by random computer selection. Because the capture probes were only 9 nucleotides in length, any one probe is expected to occur (on average) once every 131,000 bases in any double-stranded genome (once every 4^9 bp = 262,000 bp; 131,000 bases in a double-stranded sequence). A computer program was written to perform the following screens: any repeated sequence was less than 4 nucleotides; there were no terminal, 3-nucleotide inverted repeats (hairpins); any probe containing a GGGCCC repeat was discarded; G + C content was maintained between 44% and 55%. From this analysis, we randomly selected 192 probes for initial studies. In addition to the nonamer capture probes, the prototype array contained a Cy3-labled, quality control (QC) probe that served as a positional reference point and positive control for array detection. Our most recent microarray was based on 10-mer capture probes in response to the more complex salmonid genome and was used in our later salmonid fingerprinting experiments whose results are presented herein.

Microarray Fabrication. Amine modified oligonucleotides were printed on 6 – well Teflon masked slides (Erie Scientific, Portsmouth, N.H.) as previously described (9). Briefly, slides were prepared for printing by washing in 2% Microcleaner and rinsed with distilled water. The slides were washed in 3N HCl and 3N H_2SO_4 for 30 min each. Slides were thoroughly rinsed with distilled water, dried with compressed N_2, coated with 2% v:v epoxysilane (3 – glycidoxypropyltrimethoxysilane, Aldrich, Milwaukee, WI) in methanol for a minimum of 30 minutes, rinsed with 100% methanol and immediately dried with compressed N_2. Oligonucleotide capture probes were resuspended in reagent grade water and the concentration of each was measured, in triplicate. Subsequently, capture probes were diluted to 80 – 100 µM in 0.01% SDS, 50 mM NaOH print buffer. Probes were printed with an Affymetrix 417 Pin and Ring™ arrayer (Santa Clara, CA), with two complete replicate microarrays contained within each well of a 6-well, Teflon-masked slide (192 probes + several QC spots). After printing, slides were baked for 30 minutes at 130°C and stored at room temperature. Each print lot was checked for spot consistency by staining a subset of slides (6 from a print lot of 42) with SYBR Green and imaging with the microarray scanner. Printing errors are therefore identified before a microarray was used for salmonid fingerprinting.

Optimized Microarray Hybridization procedures. Typically, 20 µL of Cy3-labeled PCR products were diluted to 70 µL in hybridization buffer to achieve a final concentration of 4X SSC, 5X Denhardt's solution. Amplification products were heat denatured for 5 min at 95°C, snap cooled on ice, and divided evenly between two replicate wells. Thus, the microarray tag was generated from 8 replicate hybridization reactions (2 PCR x 2 independent hybridizations per amplification x 2 microarrays per hybridization). Denatured amplicons (in hybridization buffer) were hybridized overnight at 4°C, and

washed five times in an ice – cold solution of 4X SSC. Slides were air dried and imaged directly with an ArrayWoRx microarray scanner (Applied Precision; Issaquah, WA).

Statistical Analysis and Algorithms: Salmonid identification requires generation of reproducible fingerprints through multiple data collections, coupled with statistically rigorous algorithms to compare a tag of "unknown" origin to those of known origin. We have developed a set of algorithms in Matlab (The MathWorks, Inc., Natick, MA) to estimate the profile of an individual array (vector of spot presence/absence), combine replicate profiles to form an individual-specific tag, and compare the similarities/differences between tags. The ArrayWoRx microarray scanner software package provides estimates of spot intensity, variability in spot intensity (standard deviation), local background intensity and variability in local background intensity (standard deviation). At this time, we are converting the Matlab and ArrayWorRx-like algorithms to JAVA and ImageJ (National Institutes of Health) to provide an open, device-independent software suite for microarray analysis.

For each spot, spot and neighborhood background intensity are initial estimated and then quality-scored. For each spot meeting quality criteria, the difference in the natural logs (ln) of the spot intensity and the local background intensity is divided by an estimate of the variability in the logged background intensity (analogous to the ln(Cy3/Cy5) ratio commonly used in two-color expression profiling microarray experiments). This transformation of the spot intensity takes into account the variability imposed by the printing process and variations in nonspecific hybridization and imaging effects as captured by the local background. If the hybridization intensity of a spot is weak or absent, the transformed value should be very close to zero; otherwise the transformed value will be sufficiently greater than zero.

To determine whether a spot should be considered "ON" or "OFF", the transformed spot intensity is compared to a threshold from the standard normal distribution, spots measurements exceeding the threshold are declared "ON" and all other spots are declared "OFF". Figure 5 demonstrates this process for one glass slide containing twelve arrays. The colors in 5A represent the raw spot intensities and the white spots in Figure 5B represent the spots declared "ON". Although we recognize there are methods to account for the analytical variability in the fingerprinting process other than solely through the use of local background, our results demonstrate the ability to generate reproducible microarray tags in an automated fashion with the local background approach.

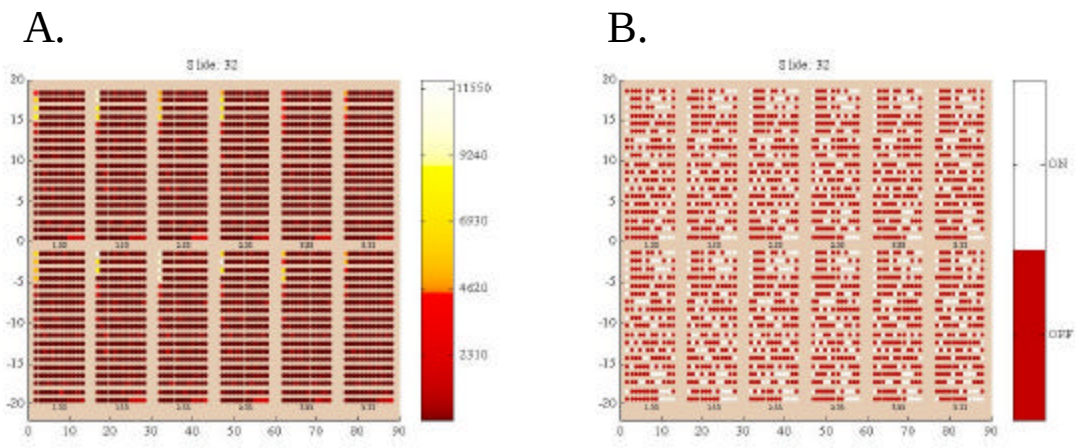

Figure 5. Estimates of spot intensity for three fish and 4 replicate arrays (A), reduced through statistical testing to an image of ON/OFF parameters (B).

Once spots are identified as "ON" or "OFF", the replicate microarray profiles for a fish are combined to form an individual-specific fingerprint estimate (Figure 6A). To reduce the fingerprint estimate to an even more robust tag or ID, we adopted a hypothesis-testing approach and assume that the proportion of spot ON determinations was based on a binomial random variable. For this phase of the analysis, the null hypothesis was that any probe spot will be observed in a certain proportion of replicates due to chance alone; for our preliminary studies, we used p=0.25. If the "ON" proportion was significantly greater than the expected chance proportion (e.g., p=0.25), the hypothesis was rejected and the spot was included in the fish tag (Figure 6B). If the hypothesis was not rejected, the spot was considered to be absent from the tag. The reduced set of tag spots was representative of the probe spots that can be confidently expected to occur when a new array profile was generated from the same fish on a different day. Thus, a series of statistically robust tags was compiled (Figure 7), and the similarity/differences between tags was then be visualized through a combination of standard similarity measures and multivariate statistical techniques (61).

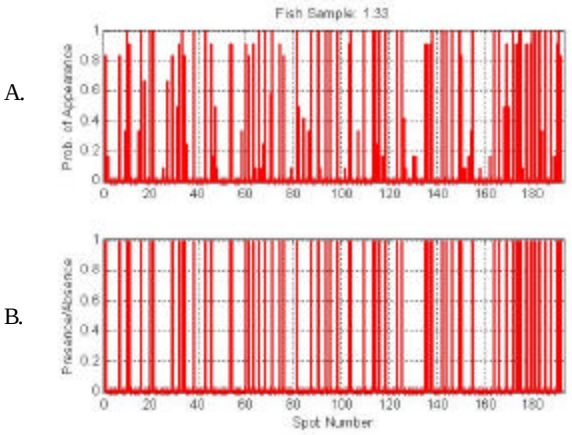

Figure 6. Microarray frequency histogram (A) based on replicate PCR amplifications and replicate microarray hybridizations, which is reduced through PNNL algorithms and statistical testing to a barcode of informative spots (B).

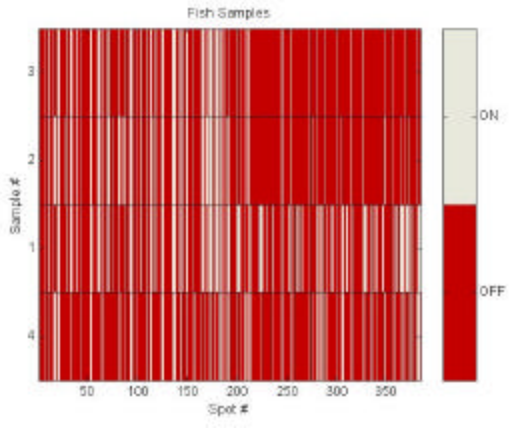

Figure 7. Individual-specific, microarray fingerprints generated from the Jeffreys 33.15, OMY77 primers and the 200-probe, prototype fingerprinting chip. The microarray data was converted to a synthetic gel image solely for the purposes of illustration.

Semi-Automated Image Analysis: The statistical models and algorithms embedded in

our image analysis tools accommodate the uncontrollable variation in microarray geometry, probe spot location and size due to the printing process, and variations in fluorescent intensity from nonspecific hybridization, local and global background noise, and stray light. Microarray imaging itself is susceptible to these errors (47), and these imaging (optical) artifacts can be easily confounded and exacerbated by image analysis software and result in erroneous spot identification, erroneous measures of spot intensities, and resulting errors in on/off declarations for each probe in the array signatures; hence, inaccurate tag estimates.

Although not currently automated for high-throughput microarray processing, we have already developed a semi-automated method for determination of spot presence/absence within a microarray image. With our current software, slide images are exported as 16-bit uncompressed *.tif files for data analysis. First, the expected print layout is warped to the actual layout observed in a printed grid, resulting in an array template. Corresponding spots in the expected and printed grids are identified using a relatively small number of mouse clicks. Then, the general row and column spacing of a printed grid is estimated using a linear model and least squares estimation. The warped grid is fit automatically to each array in each slide image using a second linear model that accounts for variation in displacement and orientation across arrays. Once the expected locations of probe spots are identified within each array and slide image, we use the BATTELLE-proprietary APEX (Automated Peak Extraction) algorithm (24) and Matlab (The MathWorks, Inc., Natick, MA) to estimate the above-background pixels at each expected spot location and the degree of reactivity (i.e. spot intensity).

A key feature of the APEX algorithm is the semi-automated identification of each spot's "above-background" pixels using a stochastic model and a statistical hypothesis-testing framework, which allows for variation in spot location, shape and intensity on the array. Under the APEX model, all pixels in the neighborhood (including those of the expected spot) were hypothesized to be background pixels of nominally uniform intensity. The APEX test statistic tests the hypothesis of neighborhood pixel uniform intensity. A spot was called "ON" if pixels in the expected spot location were more intense than adjacent pixels, so that the hypothesis of a uniform neighborhood was rejected; otherwise, the spot was deemed "OFF". The estimated "ON" spot intensity within the expected spot location was an indicator of the level-of-hybridization, and was deduced even in a highly variable local or regional background. The set of APEX-estimated spot intensities and "ON/OFF" results was ordered by probe ID for each array, and constitutes an estimated array signature. For comparison, spot intensities were also estimated with Phoretix array software (version 1.00, Phoretix International, Newcastle, United Kingdom). Replicate microarray signatures were then combined to form an individual-specific tag as described above.

Figure 8 shows a false color image from a 47-probe fingerprinting array and the attendant measures of signal intensity, illustrating the rationale for developing the APEX algorithm for the DNA fingerprinting application. For example, region 1 encompasses two probe spots where there is no visible hybridization. The APEX algorithm correctly identified these as non – hybridized spots (Figure 8B). However, the commercial software not only indicated a positive hybridization in these areas, but the estimated signal intensity for

these two spots varied by an order of magnitude (172 and 1,999 relative light units). Region 2 shows two positive probes with obvious differences in signal intensity. In this case, the commercial software assigned a signal intensity of 30,020 to the more intense spot, but a signal intensity of 31,686 to the obviously weaker spot. In contrast, the APEX algorithm assigned signal intensities of 3,483 and 2,753 (respectively), values that are at least consistent with expectations based on the raw image (Figure 8A). A similar situation is shown in regions 3 and 4 of Figure 8. Image analysis with the BATTELLE APEX algorithms therefore shows that, if we blindly use commercial (or "closed source") software to analyze and quantify microarray signal intensities, we may erroneously declare probes to be "ON" and contributing to the overall tag estimate when they are in fact "OFF". Thus, continued development or exploration of alternative "spot extraction" or image analysis tools may be required to reduce the variability associated with the fingerprinting method (see Task 3).

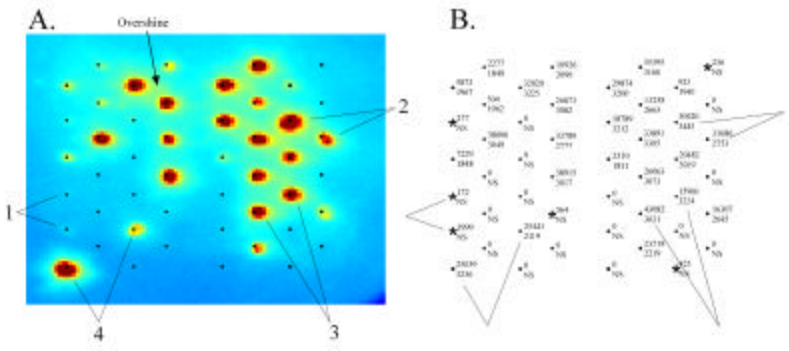

Figure 8. False color image for one fingerprinting array (A) and probe spot intensities (B) measured by Phoretix (top value) or APEX (bottom value). Overshine is visible as a green haze in panel A. Asterisk symbols in panel B indicate erroneous Phoretix on/off determinations. NS = no identifiable "on" spot using the APEX algorithm

Data Processing and Management: The power of DNA microarrays lies in the ability to simultaneously interrogate the genome at multiple locations, which naturally leads to an enormous quantity of data (57). To complete the suite of salmonid fingerprinting tools, a protocol for data handling and data management will ultimately be required. The data management toolkit should include the raw data (e.g. images), slide characteristics and metadata (batch, slide number, etc.) and other relevant information. In preparation for this proposal, we converted some of our custom statistical algorithms into "plug-in" modules compatible with ImageJ, freeware inspired by National Institutes of Health's NIH-Image. We believe that contributions to open-source software such as ImageJ, is essential for the microbial fingerprinting array and method to be broadly distributed and transferred to the user community. Continued development of the ImageJ plugins for data acquisition, analytics, and management is therefore included as one sub-task for this proposal. Further design and development of this toolkit will be made in the spirit of and under the guidance of the Microarray Gene Expression Data Society (MGED).

MGED *is an international organization of biologists, computer scientists, and data analysts that aims to facilitate the sharing of microarray data generated by functional genomics and proteomics experiments. The current focus is on establishing standards for*

microarray data annotation and exchange, facilitating the creation of microarray databases and related software implementing these standards, and promoting the sharing of high quality, well-annotated data within the life sciences community. A long-term goal for the future is to extend the mission to other functional genomics and proteomics high throughput technologies. We have included MGED principles in the design of our open software and, when possible, have incorporated their open software modules.

Summary. The proof-of-application results described here represent important first steps to high-resolution salmonid DNA fingerprinting with microarrays. With these preliminary studies, we have demonstrated the potential power of the microarray method, even though we used a very simple, 200-probe chip. Increasing the number of capture probes to 1000 or more will provide an additive increase in useful (discriminatory) data. In addition, amplifying genomic DNA with additional repetitive PCR primers will provide a multiplicative increase in effective tag probes without adding any more capture probes to the array or complexity to the tag/data analysis method.

The value of the microarray fingerprinting technique described here ultimately lies in understanding, modeling and capturing the variability in the entire experimental process. Management applications of microarray fingerprinting require a better understanding of the requisite experimental replication to ensure that the fingerprint is representative of individual- or stock-specific profiles collected on different days, with slides printed from different batches, etc. Without proper replication, observed differences between "unknown" samples and a tag library will be confounded with sources of variability inherent to the experimental process, and a reliable identification of salmonid origin may not be possible. In addition, application of the fingerprinting array to fisheries management questions will require 1) a more thorough understanding of natural (within-stock) variability in microarray tags, 2) improved analytical methods to improve sample quality and signal-to-noise ratios, and 3) enhanced, open-source image analysis software for unambiguous feature extraction and generation of microarray tags that can be transferred to the fisheries user community.

The purpose of this proposal is to demonstrate relevant proof-of-application by generating individual-specific genetic tags for up-migrating steelhead, reconditioned kelts, and their progeny. We further propose a collaborative study comparing microarray results to microsatellite gel results from the same samples generated by Columbia River Inter-Tribal Fish Commission. These results will illustrate how the microarray platform can rapidly identify individuals in the absence of a physical tag, and ultimately provide the technological basis for managers to make near-real time decisions on fisheries management issues with genetic (chip-based) stock identification data.

Significance. The CBFWA 10-year plan (1) identifies two specific areas where DNA microarray technology and tag database will provide significant impacts on fish protection and enhancement. First is the Anadromous Fish Production Construction program with its goal of retrofitting existing hatchery facilities to operate as conservation hatcheries supplementing ESUs or for captive brood stock management. Quick turn-around, accurate information on the genetic identities of potential brood stocks is

essential for operating these hatcheries in the ESU supplementation mode. The proposed DNA microarray/tag database system can be used for this operation.

Second is in the configuration and operation of the mainstem hydropower system. One of the basic issues of concern is whether and to what extent specific stocks are impacted by project passage. To date, data on this issue have been difficult to ascertain due to the expense and biological impact of tagging every out-migrating salmonid. More than 40 million salmon are physically tagged with coded wire tags and over 1 million are tagged with PIT tags every year. Despite these efforts, physical tags have not addressed the fundamental genetic or biological uncertainties associated with hatchery operations or system survival.

The DNA microarray technique could supplement the tagging programs to address questions related to hatcheries and system-wide survival. The results of the research proposed here include a DNA-based tool and associated data handling method for augmenting other studies and understanding important fisheries questions related to:

- The genetic effects of hatchery versus wild salmon interbreeding and habitat use
- Intra-specific and inter-specific ecological and genetic interactions of managed populations
- Precise definition of migration pathways and homing tendencies
- Stock or subpopulation utilization by sport or commercial fisheries
- Salmonid stock structure and populations in nature.

Relationships to Other Projects. We will be working with Dr. David Fast of the Yakama Nation to review the objectives and project data resulting from the proposed tests. We will perform these reviews to help ensure that the objectives and data answer questions related to steelhead management and interactions of wild and kelt populations. We expect to relate the results of proposed DNA microarray study to some of the specific goals of the Yakima Klickitat Fisheries Project (YKFP). The YKFP is a supplementation project designed to use artificial propagation in an attempt to maintain or increase natural production while maintaining long-term fitness of the target population and keeping ecological and genetic impacts to non-target species within specified limits. The Project is also designed to provide harvest opportunities. The purposes of the YKFP are to enhance existing stocks of anadromous fish in the Yakima and Klickitat river basins while maintaining genetic resources; reintroduce stocks formerly present in the basins; and apply knowledge gained about supplementation throughout the Columbia River Basin. Fish that we studied in preparation for this proposal were collected from YKFP facilities in Prosser and Cle Elum, Washington.

We expect to establish collaborative relationships with Paul Anders, Doug Hatch, Shawn Narum and others from CRITFC. These relationships will be the basis for sharing samples and comparing microarray and gel-based fish-fingerprinting technologies. If this microarray technology proves as successful as the authors believe, we envision this technology being adopted by CRITFC. The establishment of collaborative, collegial

relationships will lead to improvements in the applications of both techniques and statistical methods to address salmonid management issues.

The advancement of individual and stock identification techniques for management of hatchery and wild population issues is as important today and maybe more important than it has ever been. Additionally, individual and stock identification techniques for other fisheries management issues are important to many or most of the objectives in the Fish and Wildlife Plan. Some examples include:

1. In Sections 7.0 through 7.5, the Council calls for immediate efforts to gather data on wild and naturally spawning stocks, review impacts of the existing hatchery system…Review current efforts for conserving genetic diversity within and among Columbia River Basin salmon and steelhead stocks [and a] process of devising the best strategies for restoration of depleted populations of threatened and endangered species… require[ing] rigorous integration of genetics, evolutionary biology, demography and…
2. Section 5, "JUVENILE SALMON MIGRATION" which states "The failure of the region to develop better information in this area has been due in part to the unavailability of new techniques and technologies..."
3. Section 6, "ADULT SALMON MIGRATION" which states "conduct various evaluations and studies to improve the effectiveness of passage facilities and, ultimately, the survival of adult salmon and steelhead."
4. Section 7, "COORDINATED SALMON PRODUCTION AND HABITAT" which states "An ecosystem approach to species recovery requires close coordination of habitat and production measures…[to]…ensure that habitat and production measures are driven by the needs of specific populations…"
5. Section 8, "SALMON HARVEST". Our proposal relates to specific elements and statements within the Biological Opinion for Operation of the Federal Columbia River Power System. For example:
    - Throughout [the] biological opinion, NMFS uses the term Evolutionarily Significant Unit (ESU) to define anadromous salmon and steelhead populations either listed or being considered for listing under the ESA. An ESU is a population that (1) is substantially reproductively isolated from con-specific populations and (2) represents an important component of the evolutionary legacy of the species.
    - Viable salmonid populations are independent populations that have a negligible risk of extinction due to threats from demographic variation (random or directional), local environmental variation, and genetic diversity changes (random or directional) over 100 years.
    - For all other ESUs, all currently defined populations should be maintained to ensure adequate genetic and life history diversity, as well as the spatial distribution of populations within each ESU.

The advancement of genetic information that has a statistical rigor for enduring both technical and legal challenges will help in attaining these goals and others within the BiOp. Equally important, the DNA fingerprinting chip and associated statistical methods

proposed here are generally applicable to *any other species* or evolutionarily significant unit (ESU) of relevance for the management of Columbia Basin fish and wildlife.

Objectives. The goal of this project is to take advantage of recent advances in DNA microarray (or DNA chip) fingerprinting technology to address fish protection and enhancement. Specifically, we intend to apply DNA microarrays to questions related to kelt management and interactions of wild and reconditioned-kelt populations. Within the project scope, we will:

1. Develop a fingerprint library of returning adults, kelts and smolts from Yakima river samples submitted to CRITFC.
2. Analyze the library for genetic similarities and differences between fish within a stock and across stocks. Then define fish-specific and, possibly, stock-specific DNA fingerprints.
3. Perform a "blind" study showing accurate identification of an "unknown" fish to itself, parents or progeny, illustrating how the microarray platform can rapidly identify and relate individuals in the absence of a physical tag.
4. In a collaborative study with CRITFC researchers, compare microarray and gel fingerprinting methodology
5. Use fingerprint information to make inferences about the reproductive success of reconditioned kelt.
6. Package microarray fish-fingerprinting methodology in an open, accessible format, and, possibly, transfer this methodology to CRITFC.

The outcomes of this research include proof-of-application for salmonid identification, a prototype salmonid-focused DNA fingerprinting chip technology, and fish fingerprinting methodology that can be developed into a standardized fisheries management tool. The timeline associated with achieving these objectives is illustrated in the following Table.

Application of DNA Fingerprinting Microarrays and Statistical Analysis for Salmonid Identification and Evaluation of Reproduction Success.																
Task	FY 2003 Qtrs				FY 2004				FY 2005				FY 2006			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1. Lib. Gen.			**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**		
2. Lib. Anal.				**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**		
3. Blind Study						**x**	**x**	**x**	**x**	**x**						
4. Coll. Study									**x**	**x**	**x**	**x**	**x**	**x**		
5. Rep. Infer.							**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	
6. Pack &Trns											**x**	**x**	**x**	**x**	**x**	**x**
7. Report				**x**				**x**				**x**				**x**

Tasks and Methods.

Task 1. Library Definition and Generation. We will acquire a sample of tissues from the adults, kelts and progeny (i.e., F1 and F2 generations) gathered by YKNP via CRITFC. Nucleic acids will be extracted from the individuals as described in detail above. PCR

amplifications with Cy3-labelled primers will initially follow conditions described above. In addition, numerous other repetitive DNA elements have been utilized for DNA fingerprinting in salmonids, and we have preliminary microarray data showing successful tags with CAC_5, Alu repeat, M13, BmK, SNAP, Jeffrey's 33.15, Jeffrey's 33.6, SINEs, OAT18, OMS1 and OAT24 (3, 15, 26, 27, 29, 33, 35, 43, 45, 51, 53) (not shown). DNA hybridizations and data analysis will proceed as described above in the Preliminary Studies section. We will also include salmonid samples gather previously from YKNP as outliers and additional test samples for algorithm and methods development while we extract nucleic acids and process the F1/F2 animals.

Our baseline protocol for extracting and deducing salmonid fingerprints is articulated in the Preliminary Results section. At a minimum, the information contained in the tag should include the random probe sequence, the probability that the probe is "ON", an estimate of the variability in this probability, an estimate of the level of hybridization that is standardized to permit day-to-day comparisons, and an estimate of uncertainty in the level of hybridization. Before developing the full fingerprint library for the adults, reconditioned kelts and smolt, however, we will rigorously assess the performance of our current algorithms and models on a statistically-designed reproducibility study to characterize the extent and sources of experimental variation. That is, in order to make legitimate comparisons between library fingerprints and future samples, the fingerprints must be representative of a fish's signature on any given day. In addition to the sources of variability in the biological dimension and protocol, there are inherent sources of variability in the microarray and imaging processes that cannot be completely removed when making day-to-day comparisons.

The actual contribution of these sources of variability to the individual fingerprint will be identified and, when significant, included in the definition of the fish tag. If these sources of variability are not accounted for in the fingerprint, they will limit our ability to use a library for individual- or stock-specific identification in future samples. Using the experimental protocol described in the Preliminary Studies section, we will conduct a statistically-designed study with Adults, kelts, and smolts to identify the remaining sources of variation, determine how many replicates are needed and how the replicates should be collected (i.e., across multiple slides, multiple probe preparations etc.) to produce a robust, reproducible tag that can then be (quantitatively) compared against a tag library.

Preliminary data from our 200 probe 9-mer array pointed to the need to 1) include more probes on the array, and 2) increase the length of the probes in order to achieve the level of discrimination we will need to successfully differentiate among fish and to identify parents with progeny. A complete decamer library of 1,040 probes from Sigma Genosys (Woodlands. TX) is available. They have performed all pre-screening to remove pallindromic, hairpin, low GC, and other problematic probes. We will have these probes modified with a 5' amine and C18 spacers to enable covalent attachment of the probes and increase the distance of the 10-mers from the surface of the glass slide, respectively. The latter modification has been shown to increase signal to noise ratio in microarray data (58).

If sufficient differentiation cannot be achieved with these probes on our standard, planar microarray surfaces, we will use gel-pad array technology developed at Argonne National Laboratory to move probes off of the surface and into a native, solution-phase environment. With gel-pad array technology, we expect to gain additional benefits including 1) more consistent print quality, 2) near solution phase hybridization kinetics which should further increase signal to noise ratio, and 3) the ability to perform melt curve analysis of hybridized probes. This latter benefit is not immediately apparent, but one that we believe will be key to resolving minor differences in genetic tags that would allow differentiation between salmonids and determination of paternity. For each pattern of hybridized probes observed, for example, we do not know with absolute certainty if the hybridization event is a "perfect match" or mismatched by one or more nucleotides. Some mismatched tagets may hybridize to the array because of the relatively cool hybridization temperatures used in these fingerprint analyses and low stringency of the hybridization buffer. Melt curve analysis would indicate which probes are a perfect match vs. a mismatch, thereby providing a more robust measure of "ON" or "OFF" assignments that may resolve minor differences between individual- or stock-specific fingerprints.

Task 2. Library Analysis. The iterative process of library testing/refinement is described in the background section and Task 1. Current research is focused on the development of an algorithm to compare and classify (or fail to classify) the tag from a single or "unknown" fish to a library of tags for "known" fish. We propose to extend this research by using an algorithm published by Jarman et al. (23) originally developed for comparing mass spectral tags. For this phase of algorithm development, each spot in the microarray tag is treated as a binomial variable. The estimated proportions of spot presence (i.e., Figure 6A) are then used to weight the importance of each spot for comparison against the library. The null hypothesis is that the "unknown" fish (or tag) has the same origin as one of the tags in the "known" library. Assuming independence of spots, a probabilistic coverage under the null hypothesis is calculated, a threshold is applied to this probability, and the presence/absence of the unknown tag within the database library can be determined. Complete development of this algorithm for salmon DNA fingerprinting will allow us to perform the blind study proposed in Task 3.

Task 3. Blind study and salmon identification. Once the number and configuration of replicate hybridizations is deduced (Task 1), we will then conduct a limited "blind" study with a sample of F1 and F2 generation steelhead and salmonid outliers from previous work to determine if the existing algorithms (described above, and in (23)) can correctly identify or classify each fish or relate F2 to F1. The results of this study will either confirm our ability to use the current microarray fingerprinting protocol and algorithms for salmonid stock identification, or suggest possible shortcomings that need to be reinvestigated (e.g., additional replication of arrays, different protocol for replication, algorithm adjustments). One potential refinement, for example, may include the use of alternative spot extraction algorithms (i.e., APEX) that are less sensitive to imaging artifacts than our commercial software (Applied Precision).

Task 4. Collaborative Study. Once the salmonid fingerprint library is well-defined and sufficiently poplulated, and the supporting analysis and discrimination algorithms have been adapted, we propose to collaborate with CRITFC researchers to execute study comparing microarray and microsatellite gel methodologies as salmonid fingerprinting and paternity-testing tools. Though the study is yet to be precisely defined, we expect that the study will evaluate both the technical and processing performances of each method. It is expected that the study may show the two methods offer complementary information.

Task 5. Inferences about the reproduction success of reconditioned kelts. As the library grows with the yearly addition of new fingerprints, we will analyze this library in close collaboration with our YKFP colleagues to make inferences about the reproduction success of reconditioned kelts. Once sufficient samples have been gathered, this analysis will continue throughout the life of the project. We intend to use the BATTELLE's extensive statistics capability to its fullest in order to define and then make valid inferences. This effort will include the estimation of uncertainities in the results and confidence in the inferences.

Task 6. Methodology packaging and transfer. A major effort in the final year of the project will bet the packaging of the methodology in an open, accessible format, and possibly, the transfer of this methodology to our CRITFC colleagues. The methodology package will include the design of the salmonid-specific microarray, microarray analytical protocols, fingerprint generation, and paternity identification. It will include the salmonid fingerprint library and library-specific software tools. If a transfer site is identified, we will work with these individuals to ensure the effective transfer of the technology.

Task 7. Interim and final reports. A final report will be prepared describing the results of this research and the ability of DNA fingerprinting microarrays to be applied to the fundamental genetic issues that underlie fish protection and enhancement. Specifically, we intend to apply DNA microarrays to questions related to the management and interactions of wild and reconditioned kelt populations. We will articulate the relative strengths, weaknesses and applications of DNA chip technology to fisheries management and hatchery operations. We anticipate at least two peer-reviewed publications arising from this work, to be published in journals such as *Can. J. Fish. Aquat. Sci.*, *J. Fish. Management*, and *Trans. Amer. Fish. Soc.* Results from this work will also be presented at regional and national meetings as the opportunities arise. We anticipate developing additional statistical tools that are a component of an integrated fisheries management system that is tailor-made for the region.

Qualifications of Participants.

Dr. Darrell P. Chandler will be responsible for the design, development and execution of the fingerprinting microarray for salmonid stock identification, working closely with Dr. Straub and BATTELLE's molecular biology and microarray team. Dr. Chandler will jointly manage this project with Mr. McKinstry.

Dr. Chandler's current research program provides > $2.5 M in leveraged and in-kind microarray research, with grants from the U.S. Department of Energy and the U.S. Environmental Protection Agency. He will devote 10% of his time to this project, and will be jointly responsible (with Mr. McKinstry) for administrative and reporting requirements. Section Manager and Technical Group Leader, Biochip Technology Center, B.S., Biochemistry, Michigan State University, 1988, M.S., Fisheries, University of Washington, 1990, Ph.D., Microbiology, Washington State University, 1996

Dr. Darrell P. Chandler is Technical Group Leader and Section Manager at Argonne National Laboratory, specializing in molecular biology and technology development for environmental biodetection applications. He recently joined ANL after 13 years at BATTELLE, where he managed 12 projects and a multidisciplinary staff in molecular biology, chemical sensors and microfluidics, statistics, environmental microbiology, ultrasonics and analytical chemistry. He has extensive experience developing nucleic acid purification and detection methods using nucleic acid hybridization on DNA microarrays and microparticles; quantitative PCR and RT-PCR techniques, including TaqMan PCR. Current research is focused on the development of novel microfluidic platforms and reagents to enable integrated biodetection systems to be deployed in the environment and at the point of use. These efforts also include the development of biochip array technology (planar and suspension systems) for the on-line detection and characterization of nucleic acids from environmental samples.

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14.

Mr. Craig McKinstry is an applied environmental statistician with a significant salmon fisheries experience. Exploiting this experience, Mr. McKinstry will lead the statistics team and be responsible for the efficient and effective use of BATTELLE's statistics resources. He will also be responsible for project management and reporting. Mr. McKinstry will be aided in this task by Dr. Chandler and Mr. Neitzel.

Mr. McKinstry has a B.S. in mathematics from the University of Alaska Fairbanks, and an M.S. in Quantitative Ecology and Resource Management from the University of Washington in Seattle. Before coming to the Pacific Northwest National Laboratory (BATTELLE), Craig held positions with the Alaska Dept. of Fish and Game in salmon fisheries research and management, and the Duke University Cancer Research Center. At BATTELLE, Craig has provided a lead role in developing statistical methods for analyzing and interpreting hydro-acoustic data of fish behavior for both juvenile and adult salmonids. His other research interests include environmental fate and transport studies, toxicology, and mark-recapture studies.

Mr. McKinstry relevant experience includes:
Research Scientist. Battelle/Pacific Northwest National Lab, Richland, Washington. March 1, 2000 to present. This is a joint appointment between the Statistics Resources Group and the Ecology Group. Provide lead role in statistical aspects of environmental research projects. Primary areas of interest include: hydoracoustic studies in adult and juvenile salmon research, bioassay, toxicology, environmental fate and transport studies, experimental design in environmental impact assessment and toxicology studies. Other projects include: inventory estimates of radio-nuclides in nuclear waste storage tanks, statistical characterization of hand-writing. Extensive programming in SAS, S-plus/R, MatLab.

Biometrician I. Alaska Department of Fish and Game, Juneau, Alaska. Jun-15-93 to Nov-3-93, full time. Provided statistical and computing support and consultation for salmon fisheries research and management. Designed implemented and tested salmon fishery forecasting models. Main statistical methods used: generalized linear models, survival analysis, Bayesian inference, Gibbs sampling, mark-recapture population estimation. Participated in observer validation studies using mark-recapture methods on

riverine pink salmon escapement. Participated in field survey of black cod stocks in Chatham Straits, Alaska using long-line gear. Extensive statistical programming in SAS, S-plus, and SQL on an Ingres database. Designed and taught a course to professional staff in S-plus programming. Presented research at the Alaska Chapter meeting of the American Statistical Association in Fairbanks, Alaska.

Fisheries and Wildlife Technician. Alaska Department of Fish and Game.

FW Tech III: Seattle, Washington Jul-5-92 to Dec-10-92, full time. Researched and designed salmon fishery forecasting models at the Center for Quantitative Science, University of Washington, Seattle.

FW Tech III: Juneau, Alaska Jul-7-91 to Sep-30-91 and May-15-90 to Sep-15-90, full time. Technical and computing support for pink and chum salmon research. Worked with fisheries biologists and biometricians in statistical forecasting of salmon runs and population estimation from mark-recapture data. Designed and supervised the construction of two large fishwheels for use on the Chilkat River near Haines, Alaska. Trained fisheries personnel in methods for collecting, organizing and trouble-shooting large quantities of complex mark-recapture data.

FW Tech II: Taku River, Alaska May-15-89 to Sep-2-89, May-15-88 to Aug-30-88, and May-15-87 to Sep-2-87, full time. Stationed in remote field camp. Built, operated and maintained fishwheels for in-river mark-recapture population estimation of migrating adult salmon as part of a joint project with the Canadian Department of Fisheries and Oceans under the US/Canada Trans-boundary Rivers Treaty. Special projects included: multiple improvements and refinements on fishwheel construction, operation, and data management. Worked directly with NMFS staff in radio-tagging and tracking of adult Chinook and Coho salmon. Conducted spawning surveys on up-river escapement. Worked cooperatively with Canadian commercial fishers in collecting biological samples and tag recovery. Extensive use of power equipment, boats, outboard and inboard motors.

FW Tech I: various field location in SE Alaska Jun-15-86 to Sep-2-86, full time. Built and operated escapement weirs at remote sites in Southeastern Alaska. Kept daily escapement counts on five pacific salmon species, collected biological samples, lived and worked safely in close proximity to Alaska brown bears. Extensive use of power equipment, boats, and

Commercial Fisher. Kodiak, Alaska. Jun-82 to Jul-83. Worked as a deckhand on commercial fishing vessels, including purse-seine fisheries for salmon and herring, and pot fisheries for king, tanner, and dungeoness crab. Engaged in all related activities including, operation, maintenance and navigation of 40` to 96` fishing vessels and related fishing gear.

Mr. Geoffrey A. McMichael, a BATTELLE senior research scientist, has worked on fish and wildlife issues in the Columbia River Basin for 13 years. He will work with the team to review the objectives, design and execution of the specific tasks to assure that

expected results are applicable to fishery management issues. He will work with the staff to review the data that are generated by this research and assess the data's applicability to these questions.

Mr. McMichael has been working on an extensive evaluation of the effects of hydropower operations on all freshwater life history stages of the fall chinook salmon populations in the mid-Columbia River. He has also performed an ADCP survey of water velocities upstream of Grand Coulee Dam. He has also been working on a project to evaluate the spawning habitat selection by fall chinook and endangered chum salmon in the Ives Island area below Bonneville Dam. Other projects include conducting evaluations of the effectiveness of fish screening facilities in the Yakima and Walla Walla river basins and conducting an investigation of smolt losses and low water evaluations at Chandler Canal in the Yakima River Basin. Geoff's past research emphasis has been in the areas of ecological interactions between hatchery and wild salmonids, behavioral observations, fish population monitoring, fish capture methods development, predator-prey interactions, and electrofishing injury.

Publications.

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McMichael G.A., J.A .Carter, J.P. Duncan, C.L. Rakowski, J.A. Serkowski, M.C. Richmond, and C.S. Abernethy. 2002. Chandler Canal Fish Screen Low Water Evaluations 2001. PNWD-3144, Pacific Northwest National Laboratory, Richland, WA.

McMichael, G.A., D.R. Geist, T.P. Hanrahan, E.V. Arntzen, R.P. Mueller, R.A. Moursund, J.A. Carter, J.M. Becker, C.A. McKinstry, W.A. Perkins, D.D. Duable, T.M. Degerman, J. R. Skalski, R.L. Townsend, B.B. James, and D.R. Thornhill. 2003. Chinook salmon in the Priest Rapids Project. Report prepared for the Public Utility District No. 2 of Grant County. Technical Report PNWD-3243, Battelle – Pacific Northwest Division, P. O. Box 999, Richland, Washington, 99352.

Dr. Timothy M. Straub, will be jointly responsible, with Dr. Chandler, for the adaptation and application of microarray fingerprinting technology to salmonid identification and paternity testing.

Dr. Straub, a BATTELLE senior research scientist, has focused on developing DNA microarray technology for organism fingerprinting. In particular, he has developed this technology for multiplexed detection of pathogenic microorganisms in water and wastewater. He works within a multi-disciplinary team environment that includes biochemists and engineers. This team focus is to develop next generation autonomous air and water quality monitoring methods for natural and introduced microbiological threat agents in the environment. Prior to employment at Battelle, he was a contractor for the United States EPA and was responsible for designing and implementing the protocols for producing and shipping protozoan performance evaluation samples under the Information Collection Rule.

Publications.

Straub, T. M. and D.P. Chandler. 2003. Towards a unified system for detecting waterborne pathogens. J. Microbiol. Meth. *In press, proofs available upon request.*

Kingsley, M. T., T. M. Straub, D. R. Call, F. J. Brockman, D.S. Daly, S. C. Wunschel, and D. P. Chandler. 2002. Fingerprinting closely related *Xanthomonas* pathovars with random nonamer oligonucleotide microarrays. Appl. Environ. Microbiol. 68(12): 6361-6370.

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Mr. Duane A. Neitzel, Staff Scientist for Battelle at the Pacific Northwest National Laboratory, has worked on fish and wildlife issues for the Bonneville Power Administration and other clients in the Columbia River Basin for 30 years. He will work with the team to review the objectives, design and execution of the specific tasks to assure that expected results are applicable to fishery management issues. He will work with the staff to review the data that are generated by this research and assess the data's applicability to these questions.

Mr. Neitzel, staff scientist with the Ecology Group, Pacific Northwest National Laboratory, joined BATTELLE in 1972. His research efforts have focused on fisheries issues and the assessment of impacts to aquatic ecosystems from the development and production of energy, and the management of hazardous wastes. The regulatory drivers behind many of his projects are result from NEPA requirements. He is currently editor of an annual document for the U.S. Department of Energy at Hanford that describes the affected environment and is used at Hanford for EIS/EA documents. He is also currently working with the Western Area Power Administration in Folsom, California on their NEPA activities. Mr. Neitzel has reported his work in over 100 journal articles, symposium proceedings, and technical reports. Additionally, he has managed or facilitated environmental research workshops related to hazardous-waste site management, fisheries research, arid ecosystems, and marine pollution research.

Relevant Job Completions:
1 -Yakima Fisheries Project. Mr. Neitzel managed BATTELLE's participation in the Yakima Fisheries Project. The projects included plans to build hatchery and rearing facilities for enhancing the salmon and steelhead populations of the Yakima Basin. Mr. Neitzel was involved in the long-range planning documentation, which includes

preparation of the project status report, project schedules, risk analysis, experimental designs, monitoring plans, and project reviews.

2 - Threatened and Endangered Animals. Mr. Neitzel managed an effort to assess the status of the giant Columbia River spire snail Fisherola nuttalli and the great Columbia River limpet Fluminicola columbiana. Both species were candidates for protection under the federal Endangered Species Act. Data collected during this study provided the U.S. Fish and Wildlife Service with the data needed determine the level of protection required for these animals in the Columbia River basin. The study included a survey of sensitive aquatic habitat at the Hanford Site. During 1992, an undescribed species of Cryptomatix n. sp was found.

3 - Advanced Hydro Turbine Design. Mr. Neitzel manages a project of the U.S. Department of Energy to define biological specifications for hydropower turbines. The study includes to design and operation of a laboratory facility to simulate shear, turbulence, pressure and other fluid forces that impact fish during turbine passage. The study results will be used by DOE to design fish-friendly turbines.

4 - Snake River Salmon Recovery Plan. Mr. Neitzel managed a project with the U.S. Army Corps of Engineers to provide technical assistance in support of the Corps efforts to improve survival for Columbia River system salmon populations. To date, tasks have included monitoring the impacts of reservoir drawdown to salmon redds, riparian vegetation, wildlife habitat, and benthos. He worked on a biological plan to describe the potential impacts and management implications of drawing down the lower Snake River reservoirs. The plan described affected populations, drawdown strategies, and risk management and was used by the Corps, the Bonneville Power Administration, and the U.S. Bureau of Reclamation.

Dr. Don Simone Daly, a senior research scientist of the BATTELLE Statistical Resources Group, will be responsible for microarray image analysis and process control, coordinating the algorithm development and software conversion tasks and general statistical support. Dr. Daly has been instrumental in developing new statistical methods for quantitative microarray fingerprinting.

Dr. Daly joined the Pacific Northwest National Laboratory operated by Battelle Memorial Institute March 28th, 1981. He is an applied statistician with expertise in the analysis of large, heterogeneous data sets, statistical models of instruments and measuring systems, and image/spectral analysis. He has an extensive background in chemistry, physics and engineering.

Recently, Dr. Daly has been developing image analysis algorithms and process control protocols for microarray (biochip) applications. With his collaborators, he extended their patented algorithm for spectral peak detection to multiple dimensions for application to spectral imagery time series. He has recently developed sampling procedures, analysis routines and decision protocols for the ultrasonic inspection of food products. This

development has led to a patent application about an ultrasonic level monitor for liquid-filled vessels.

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12. Daly DS. 2000. "A Method for Detection and Characterization of Biopathogens System Specification and Evaluation." In *Laboratory Directed Research and Development Annual*

Report – Fiscal Year 1999, pp. 339-340. BATTELLE-13203, Pacific Northwest National Laboratory , Richland, Washington.
13. Jarman, K.H., D.S. Daly, C.E. Petersen, A.J. Saenz, N.B. Valentine, and K.L. Wahl, Extracting and Visualizing Matrix-assisted Laser Desorption Ionization Time of Flight Fingerprints, Rapid Communications in Mass Spectrometry. 13(15): 1586-1594, 1999.
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BATTELLE Statistics Resources.

In research and development and in the application of technologies, statistical expertise is required to plan data collection activities and interpret the results of experiments. Uncertainties in the data must be quantified, constrained, or modeled to exercise proper control of a system or to ensure correct decisions and conclusions. The statisticians of the Statistical & Quantitative Sciences group (S&QS) at Pacific Northwest National Laboratory bring a unique perspective on uncertainty to multi-disciplinary teams. S&QS provide practical solutions to important problems that influence policy decisions in industry and government. S&QS is represented by approximately 30 statisticians (half PhDs and half with MS degrees) with a broad range of statistical and mathematical academic backgrounds and experience that brings to the Laboratory a diverse set of capabilities: Design, Test Plan Development, Statistical Evaluation, Sampling, Geostatistics, Data Analysis, Modeling, Simulation, Uncertainty Analysis, Data Mining, Informatics, and Chemometrics. S&QS offers a full array of statistical approaches ranging from employing existing statistical methodology to development of new statistical paradigms. Collaborations with other scientists and engineers facilitate multi-disciplinary breakthroughs at the intersections between the statistics capability and the other science capabilities.

Facilities and Equipment.

The following facilities and equipment are available at no additional cost to the project. No additional equipment purchases are required in order to conduct the proposed work.

Pacific Northwest National Laboratory. The Environmental Microbiology Group at BATTELLE has 4500 ft^2 of newly designed and built laboratory space, including a dedicated BL-2 pathogen laboratory, soils/sediment processing lab, radiological facilities, aerobic and anaerobic microbiology areas, and a molecular biology lab. We have also received CDC approval for handling anthrax DNA. Equipment currently available includes: a Genetic MicroSystems 417 DNA array printer, ArrayWorx CCD confocal array scanner; 2 Perkin-Elmer 9600 PCR thermocyclers, 1 Perkin-Elmer 7700 real-time quantitative PCR system, 1 MJ Research Tetrad thermal cycler with 4 x 96 well independently controlled sample blocks, a dedicated PCR clean room with laminar flow hood; Qiagen bio-robot; Applied Biosystems 373 and 377 automated DNA sequencers, DNA synthesizer, nucleic acid and protein sequence analysis software and databases; Bio-Rad Fluor-S imager; fluor- and phospho-imager station; 2 Sun workstations for genomics, informatics and phylogenetic analysis of DNA sequence information; DNA fluorometer, nucleic acid electrophoresis equipment including gel boxes, electroeluters, rotary hybridization ovens, Stratalinker UV crosslinker, blotting apparatus and

accessories; electroporater, luminometer, 3 bench-scale bioreactors, 5 anaerobic glovebags; light and epiflourescent microscopes, a confocal laser scanning microscope, scanning electron microscope; various shakers and incubators; high-temperature incubators; laminar flow and biosafety hoods, MIDI and Biolog systems for automated identification of bacterial cultures; protein electrophoresis equipment, amino acid analyzer, visible and UV spectrophotometers, superspeed- ultra- and micro-centrifuges, refrigerators and cryogenic storage facilities; 2 HPLCs, 2 GCs, FPLC, GC-MS, and 2 liquid scintillation counters. The Environmental Microbiology Group at BATTELLE has been funded and authorized to renovate 4 laboratory spaces in FY2003. These spaces will be dedicated to the study of biosafety level 2 and level 3 select agents. Work to renovate these laboratories is anticipated to begin within the last months of FY2002.

Within the fiscal year, the Environmental Microbiology Group will merge facilities with the eukaryotic biology group to construct and operate a microarray core facility. Equipment exists (major equipment below) or has been ordered, and will be received by the time this project begins. Merging molecular capabilities will also allow access to in-vitro cell culture facilities currently maintained by the eukaryotic cell biology group. The microarray core facility has these pieces of major equipment: Microgrid II arrayer, Environmental Microbiology Group, Capable of spotting up to 120 slides in a single run, autohandling of 15-96 or 384 well plates humidity controlled printing.Applied Biosystems ScanArray 5000, Environmental Microbiology Group, 3 laser scanner to cover the entire spectrum of dyes available, auto slide handler can scan 20 slides in a single run Affymetrix 417 arrayer, Environmental Microbiology Group,prints 42 slides per run, and has the capacity to print from 3-96 well or 384 well plates. Applied Precision ArrayWorx Scanner, uses white light and a series of excitation and emission filters to cover the entire spectrum of commercially available dyes. Capable of reading 42 slides in a single runQiagen 8000 biorobot, Environmental Microbiology Group. This is a multifunctional biorobot that can be used for purifying PCR products in microtiter plates, set up for plasmid preps, set up for producing microtiter plates for microarrays.

Argonne National Laboratory. The Biodetection Technologies Section of the Energy Sciences Division (ES) is staffed with a bioengineer, physicist, two chemists, mathematician/software specialist, several molecular biologists and microbiologists. The Biochip Group occupies approximately 4,027 sq. ft. of laboratory and 1,680 sq. ft. of office space consisting of conventional biochemical, chemical, microbiological and molecular biology areas. Separate rooms are dedicated to biochip manufacture, mass spectrometry, PCR amplification and biochip imaging. The offices are adjacent to the laboratories. In addition, the Biochip Program has completely renovated a 1000 sq. ft., CDC-registered BSL-2 suite, complete with a 14 x 24 ft Class 1000 cleanroom (National Cleanrooms, Inc.) and custom-designed Quadrate IV gel pad microarray production robot for exacting biochip manufacture, quality assurance and quality control.

Additional equipment currently available in the Biochip Program includes: Beckman Biomek 2000 liquid handling station; Quadrate II printer with integrated optical quality control; dedicated robotics room with environmental control; Genetic Microsystems 417 arrayer; Qiagen (Luminex) LiquiChip flow cytometer; 2 Microm DS50 automatic slide

stainers for microarray slide preparation and activation; Bruker MALDI-TOF Biflex III mass spectrometer powered by a Sun Ultra 5 workstation; Tecan HS4800 automated hybridization station; Packard laser-confocal microarray scanner; Oriel columnated UV light source; 2 Liquid chemical dispensing robots (LCDR) for parallel synthesis of 96 oligonucleotides each; Hewlett Packard 8452 Diode array spectrophotometer; Molecular devices SpectraMax spectrophotometer and automatic plate handler; Perkin Elmer LS50B Luminescence spectrophotometer; Perkin Elmer Lambda Bio 10 Uv/Vis spectrometer; Applied Biosystems 394 DNA/RNA synthesizer; 3 Millipore ultra-pure water systems; MJ Research Dyad with glass slide sample blocks; Eppendorf Mastercycler gradient thermal cycler; Perkin Elmer GeneAmp 9700; Stratagene Robocycler gradient 96 thermal cycler; Perkin Elmer GeneAmp 1000 In Situ PCR system; MJ research Thermal controller; several refrigerated microfuges; 3 cryogenic -80 freezers; a complete set of horizontal and vertical gel electrophoresis equipment and power supplies; Applied Biosystems 373A DNA sequencer; analytical balances; several phase contrast and fluorescent microscopes; 3 custom microscopes with integrated thermal tables; Dynamax and Waters HPLC systems with absorbance and temperature sensors and fraction collector; 2 Labonco speed vacuum dryers with 96-well plate capabilities; Buchi rotary evaporator; Pharmacia Uvicord SII; analytical balances; peristaltic pumps; several peltier thermal tables; Beckman Coulter table-top centrifuge; dedicated optics shop with complete tools, optical tables, power supplies, etc; sequential injection pumps, valves, and selection valves; refrigerated and heated water baths; various shaking and stationary incubators; and digital gel image capture and photographic supplies.

The Biochip Program has developed customized software for full automation of the robotic arrayers Quadrate II and IV. Custom-designed software was written and deployed on the fluorescence microscopes and portable biochip analyzers. The Biochip Program has approximately thirty personal computers, a Linux server, and Sun platform. The Division (Energy Systems) maintains a full-time Systems Administrator who is responsible for the managing of computer support/network connections including security updates in the Biochip Group, with additional support available through the Laboratory's Electronics and Computing Technologies (ECT) Division.

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