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PRINCIPAL INVESTIGATOR: Harold P. Swerdlow, Ph.D.

CONTRACTING ORGANIZATION: Karolinska Institute
S-171 77 Stockholm, Sweden

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Harold P. Swerdlow, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Karolinska Institute
S-171 77 Stockholm, Sweden

E-MAIL:
Harold.swerdlow@cgr.ki.se

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13. ABSTRACT ***(Maximum 200 Words)***

This project addresses the design and prototyping of a multiple-capillary (ultimately 96-channel) electrophoresis system that will be ideally suited for the rapid and inexpensive analysis of allele-specific and DNA-sequence-based breast-cancer diagnostic tests with high throughput. A fully-automated capillary electrophoresis system (as opposed to an electrophoresis instrument) will perform the following steps without human intervention: 1) prepare and purify DNA, ideally from blood or tissue samples, 2) mix DNA samples with PCR reagents, 3) thermally cycle and purify PCR products, 4) mix PCR samples with DNA sequencing reagents, 5) perform sequencing reactions (thermal cycling typically) and purify the fragments, 6) pre-run the capillaries and load the samples, 7) perform the electrophoresis and detect the DNA bands, 8) record and analyze the data, and 9) flush the capillaries after the previous run and fill the capillaries with fresh polymer solution. The present study will automate steps 6-9 of this process.

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FOREWORD

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N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Harold Swerdlow 9/20/99

PI - Signature Date

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Introduction

Well, it's been a rebuilding year. One year ago, I relocated from the University of Utah to Stockholm, Sweden. My new home is the Karolinska Institute - a widely respected hospital and biomedical research facility - the largest of its kind in Northern Europe. I was hired to assist in shaping a new department at the Karolinska, the Center for Genomics Research (CGR).

CGR was established by a large (by Swedish standards) grant from Pharmacia and Upjohn to the Karolinska Institute to help support basic and applied research, especially in the area of technology development and human genetics. CGR has evolved into a loosely connected core of eleven group leaders, with widely-varying primary research interests, but all dedicated to "genomics". At CGR, we define genomics as the branch of biological science that uses the set of highly-parallel, high-throughput macromolecular technologies of the 21st century to answer previously unanswerable questions from biology, physiology, and the study of disease. We are committed, not to obtaining the first (or subsequent) sequences of humans nor model organisms, but to use that sequence information for the elucidation of the structure and function (and dysfunction) of mammalian genes and their products. Focused efforts in mouse transgenics, antisense technologies, DNA sequencing , coding SNP identification / analysis, DNA microarrays, DNA diagnostics, and proteomics, all supported by a strong bioinformatics team, are part of this new endeavor.

At Karolinska, numerous resources exist for obtaining cell-based model system materials, and clinical samples, when they become germane to our projects. Additionally, proximity to key collaborators in the fields of spectroscopy, DNA analysis and DNA diagnostics will aid in our efforts. Specifically relevant to our Breast Cancer Research Program efforts, are:

- The Twin Registry - a complete database of all twins born in Sweden since the late 1800s. The twin registry is being expanded currently by obtaining questionnaires, health records, and even blood and DNA samples in some cases from both young and old twins born since 1920. This resource will be invaluable as a source for many types of genetic testing, and gene finding. A current study aims to create an accurate assessment of the complete genetic component of breast and other cancers in Sweden. This has long been an issue, since the discovery of BRCA1 and 2 have invited the question of how many other breast-cancer predisposition genes of somewhat lower prevalence / penetrance are there in the population.
- The Swedish Family Cancer Database - a subset of the Swedish Cancer Registry, the latter containing reference to essentially all individuals born in Sweden since 1941 (6 million persons). The Family Cancer Database has approximately 30,000 cancers registered in total, including many breast and ovarian cancer families. This database has been used, for example, to assess the relative risks of

contracting certain cancers in offspring of affected parents (Hemminki, K and Vaittinen, P. (1997) Interaction of Breast Cancer and Melanoma Genotypes, Lancet 350, 931-932.) The researchers involved are located at the Karolinska Institute and have expressed a willingness to work with the Center for Genomics Research.

- Dr. Lennart Iselius, at the Karolinska Hospital - a surgeon that specializes in breast-cancer surgery and genetic analysis.
- Anthony Brookes, at CGR - a human geneticist, specializing in DNA diagnostics. Dr. Brookes is the inventor of a new fluorescence-based technique for SNP analysis based on dynamic allele-specific hybridization (DASH). He is interested in finding disease-genes with this technology.
- Rudolf Reigler, at Karolinska Institute - a spectroscopist with expertise in single-molecule detection and DNA sequencing.
- Mathias Uhlén, at the Royal Institute of technology in Stockholm - a world leader in sample preparation for DNA sequencing / diagnostics and the inventor of the magnetic-bead approach to DNA sequencing reaction purification.
- Pål Nyren, a biochemist, also at the Royal Institute - with Mathias Uhlén, the co-inventor of a novel luminescence-based technique for DNA sequencing, called "pyrosequencing.."
- Ulf Landegren, at the University of Uppsala - an expert in mutation detection and the enzymology of DNA-diagnostic reactions.

My role at CGR is to bring new methods and instrumentation to bear on biological problems. The capillary-electrophoresis instrumentation that I am developing under this U.S. Army Breast Cancer Research Program grant for breast-cancer diagnostics is a key element of this effort. Also of interest to the breast-cancer program, under a separate IDEA grant, is a project to develop novel instrumentation for the next generation of microarrays for hybridization-based assays. Other projects in my lab include novel microfabricated microfluidic structures for performing DNA diagnostics in a high-throughput, inexpensive format - the microchip.

I moved from the University of Utah to CGR, 17 days before the beginning of the current grant period (August 22, 1998- August 21, 1999), the first year of a two-year no-cost extension. The U.S. Army Breast Cancer Research Program and the University of Utah administration graciously agreed to allow me to transfer this grant to the Karolinska Institute. Unfortunately, due to complicated circumstances, the remaining funds from the University of Utah, as of this writing, have still not been received. Therefore, no U.S. Army monies were expended during this grant period!

Since none of the members of my Utah group moved with me, I have had a difficult time in setting-up my research activities here in Sweden. Additionally, I have been occupied this past

year in building up a modern technical research facility for my group, with wet and dry laboratory space, optics prototype facilities (darkrooms), a clean-room, numerous lasers, DNA-sequencing instruments, capillary-electrophoresis systems, machine-shop tools, an electronics assembly station, and microarray analysis instruments. I have also used this time to bring in 6 new team members, and to train most of them in the methods we employ.

These major distractions notwithstanding, we have managed in one year, to essentially retrace and reestablish our technical expertise in the field of capillary electrophoresis and fluidic sample preparation. This will now be described.

Body

Our 96-channel capillary detector is based upon fiber optic excitation and CCD-camera detection. Alignment is very simple - only one fiber needs to be aligned and then they are all aligned. No moving parts in the design insures long-term stability and robustness. The detector has now been re-assembled and the graduate student who will be performing these experiments is fully up-to-speed. We have made several small modifications during the course of this work to allow easier assembly and use. Currently, for providing excitation light to all the capillaries, we are relying upon a fiber-optic network that is micromachined commercially in a planar-glass waveguide format. This waveguide splitter is fed from a single fiber-optic input that is coupled directly to the laser. The launching optics have been redesigned. We have replaced the original microscope lens employed with a Newport model U-13X, an antireflection-coated quartz objective with a 0.13 numerical aperture. This lens provides more efficient coupling as a result of the anti-reflective coating, the quartz elements, and the better match between its numerical aperture and the numerical aperture of the fiber (0.22). With the use of better alignment holders, the overall waveguide coupling efficiency is now about 60 %; i.e., 60 % of the ingoing laser light ends up coming out of all the waveguides when summed together.

The capillary array holding strategy has been re-engineered. The previous design was tricky to align and keep in place. The new holder is easier to use and more reliable. We have also begun talks with a small company here in Sweden about manufacturing the capillary holder (and possibly the fiber alignment pieces as well) from a single block of plastic or silicon by micromachining; i.e., by using a photolithographic-based method. Such a holder would eliminate all the remaining alignment issues, and allow the entire capillary (fiber) assembly to be readily manufactured as a disposable unit.

Lastly, the holder for the holographic notch filter has been replaced. The previous design was fraught with instability. The new design is far better. These changes have now been incorporated and the system has been tested. A single-color run performed with the newest design is shown as Figure 1. Signal-to-noise is good, and resolution, although not the best we have ever obtained, is adequate for sequencing.

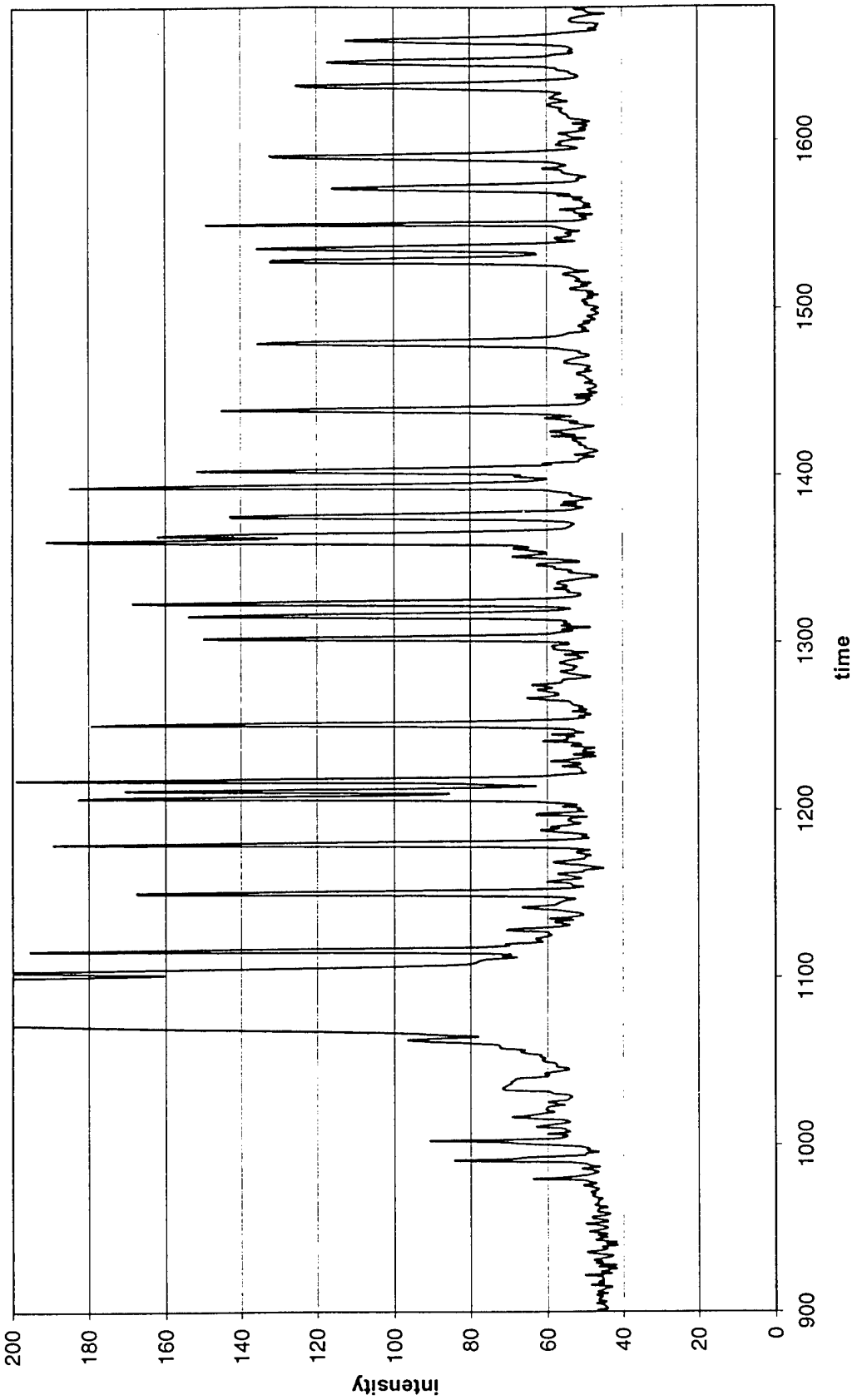

Figure 1. Capillary Electropherogram of the first portion of a DNA sequencing run performed on the 8-capillary prototype instrument. Matrix: Pop6, injection 30 sec. Run 180V/cm, length 36 cm.

We have also begun work on the fluidic-loading strategy previously described. This project was decimated by the move, in that the engineer who had been designing, optimizing and testing the fluidic-loading manifold went on to design personal-use helicopters. However, we are currently in the process of re-optimizing, and re-testing the manifold prototype. External connections to the device have been improved. The pressure supply, key for accurate loading, has been made more reliable by the addition of a regulating unit and valve assembly. The leveling devices for all supply buffer chambers has been improved as well. These are key for the electrophoretic separation. Any deviation in fluid height between various buffer chambers can manifest itself as a loss of resolution due to hydrodynamic flow in the capillary. Actual sequencing reactions are being tested at this time. We expect clear results soon, which will allow us to build the 96-channel prototype. Subsequently, we will begin the task of connecting this fluidic loader to upstream sample preparation and purification subsystems.

Key Research Accomplishments

- design and development of an automated fluidic loading manifold for capillary electrophoresis of DNA
- design and implementation of a 96-channel full-spectral detection strategy
- development of improved protocols for capillary electrophoresis sample loading
- development of improved formulations and protocols for polymer-filled capillary DNA sequencing
- development of improved fluorescent dyes for DNA sequencing

Reportable Outcomes

none this period

Conclusions

After a brief hiatus, we are ready to go forward again. Two different 96-channel capillary instruments are currently on the market, the Applied Biosystems 3700 and the Molecular Dynamics MegaBACE 1000. I believe these instruments both need further development before they are fully functional and highly efficient sequencing instruments. Preliminary indications are that poor read-length and tricky sample preparation are the two most critical issues. Although some of this development is methodological, some is also related to instrument design. Our multiple-capillary electrophoresis detector design promises to be a significant improvement over other designs, as a result of the lack of moving parts (or moving sheath fluids in the case of the 3700). Additionally, most of our work is now focused on improving sample preparation methods, via automated fluidic loading strategies. We believe this approach offers significant enough advantages over robotic loading to warrant further study.

Recently, we have become interested in analyzing complex samples. Part of the impetus for this effort comes from an inter-disciplinary project evolving at the Karolinska Institute that will attempt to analyze, in as much detail as possible, the complex flora in the gut. The methods have not yet been finalized, but the aim is to explore the interactions between disease, diet, stress-level, etc. and the total spectrum of intestinal microbes, dynamically. One scenario we have discussed involves PCR or direct analysis of fecal material. A similar project we have talked about would aim to analyze DNA in blood samples. Recent findings have shown that there is a significant concentration of tissue DNA circulating in the blood (typically 10-30 ng/ml), with a half-life long enough to detect sequences of interest by various protocols. The story gets really interesting, when blood from cancer patients is analyzed - the circulating DNA concentration jumps typically to 100 ng/ml. Preliminary experiments support the tumor-cell origin of this DNA, as the DNA in lung, kidney and prostate cancer patients showed decreased strand stability similar to tumor DNA (Bevilacqua, R.A.U., et al. (1998) The use of genetic instability as a clinical tool for cancer diagnosis. Semin. Cancer Biol 8:447-53). Although it has yet to be shown that breast cancer-specific DNA is circulating in patients with either hereditary or sporadic disease, such a finding could revolutionize breast-cancer diagnosis. Possessing this tool would alleviate our concerns that (early) DNA-based breast cancer diagnosis could only be done by routine biopsy of breast tissue, or by inference from genetic data. Since we have a number of high-sensitivity PCR and capillary-electrophoresis-based methods at our disposal, we will consider exploring this approach to DNA-based diagnostics in the near future. The usefulness of this approach for various cancers would extend to surrogate markers for therapeutic and prognostic indications - such tools offer immense benefits to both patients and the pharmaceutical industry.