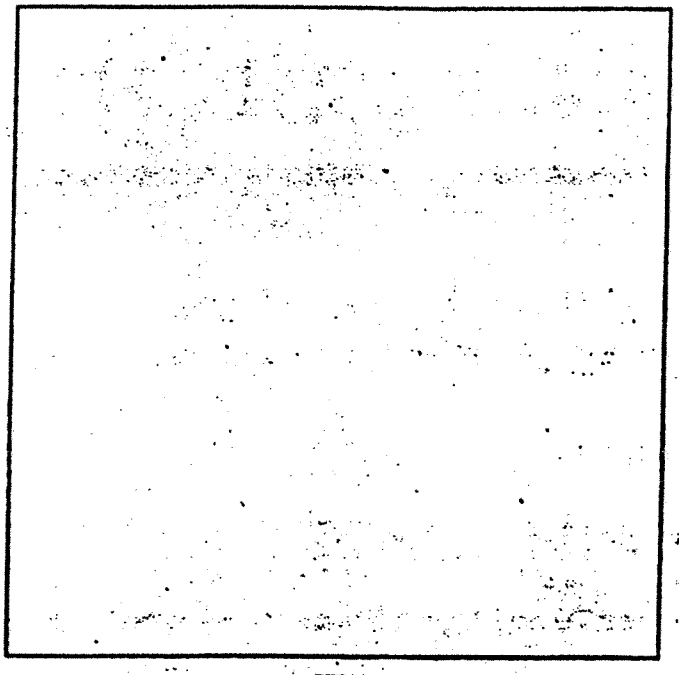
BACKGROUND DENSITY TARGET



Hoffmann-La Roche Inc.

NOTEBOOK NO. 4489
ISSUED TO Shirley Kwok
ON Feb 21 19 91
DEPARTMENT Infectious Diseases
RETURNED 19

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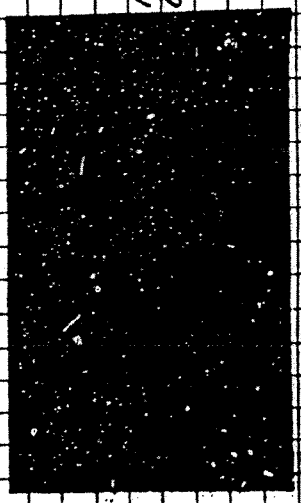
66 Project No. Book No. TITLE Generation of Shuffle Sequence

From Page No. 7/3

Andy's 420



Digested 25 ul of product c PstI/BamHI

5 ul 10x High Salt
25 ul product
1 ul PstI
1 ul BamHI
18 ul dd H2O

37°C for 2 hrs

Ran to N.M for cloning into sp6 vector

To Page No. Eng

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Date 8/6/91

Invented by

Recorded by

Date 7/3/91

TITLE Proposal for Quantitative Assay Project No. Book No. 67

From

Current Status of the HIV-2 Assay and a Proposal for Development of an HIV RNA PCR Quantitative Assay (S Kwok)

The HIV-1 and HTLV assays developed in our lab are currently being finalized at RDS. As of May 27, 1990, our lab has focused primarily on developing an assay for HIV-2. Since the HIV-2 project was initiated last year, we have encountered two major obstacles.

First, sample procurement has been a problem. The CDC has provided us with some HIV-2 samples. However, these are few and far between. Furthermore, the amount of DNA we receive for each sample is generally enough for only a few reactions which poses a problem when dealing with recalcitrant samples. We have recently initiated a collaboration with Dr. Vamier at the University of Genova in Italy. We received 50 HIV-2 samples from his group but many of these samples were not PCR competent as determined by HLA amplifications. Once again, the quantity of material was insufficient for extensive studies. The ability to obtain large quantities of well-characterized HIV-2s is absolutely critical to the success of this project. Once a good candidate primer pair has been identified, validation of the primers will require yet another batch of samples.

Second, the HIV-2 genomes are more heterogeneous than the HIV-1s and selection of a primer pair-probe system that amplifies all isolates has been a challenge. To date, sequence information is available for only 7 HIV-2s. Three SIV isolates, a virus closely related to HIV-2, have been sequenced. Despite identifying regions that are highly conserved (which are few), we have only been able to detect 80% of the isolates with our best primer pair. It appears that the sequenced isolates may represent but a fraction of those present in the population. Furthermore, HIV-2 isolates from different parts of Africa may be divergent.

Page No.

Witnessed & Understood by me, | Date | Invented by | Recorded by

Project No.
68 Book No. TITLE

From

The incorporation of HIV-2 into the HIV-1 assay has obvious appeal. HIV-2 has been observed primarily in West Africa and to date, only a handful of individuals in the United States have been infected with this virus. These individuals were either from West Africa or had contracted the disease there. Serological and peptide assays for HIV-2 are available. In addition, given the problems with sample procurement and the heterogeneity of the virus, I feel we need to reevaluate the priority given to the HIV-2s.

I propose that rather than devote the entire lab to HIV-2, that we begin to develop a procedure for quantitation of HIV-1 viral load in infected patients. Our effort on HIV-2 would be decreased but not terminated. There is a huge demand for quantitative assays to evaluate drug efficacy. A quantitative assay for HIV RNA by PCR would fill this need.

CONSIDERATIONS:

In developing a quantitative HIV RNA assay we need to take into consideration every aspect of the procedure, from sample preparation to amplicon detection

A) Selection of a sample preparation protocol. Ideally, the sample preparation protocol should be easy to use and require only a few manipulations. Microprobe's and HRI's extraction procedures are simple, but the effeciency of sample recovery needs to be assesed.

B) Reverse transcription and PCR. Reverse transcription and PCR will be performed in a single tube with the Tth DNA polymerase. To the extent possible, reactions will be pretreated with UNG and amplified in the presence of dUTP.

Witnessed & Understood by me, | Date | Invented by | Date
Recorded by

Project No._____

TITLE_____ Book No._____ 69

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possible, reactions will be pretreated with UNG and amplified in the presence of dUTP.

C) Cycling. For accurate quantitation, the range of exponential amplification as a function defined cycle number needs to be determined.

D) Detection. Ideally, the detection method should be non-radioactive, have a sensitivity of 10^7 molecules, and have a broad dynamic range (1-1000 input molecules). It would be best if a colorimetric microtiter detection format similar to that currently used at Roche could be employed. The current HRP detection method has a very narrow range (1-15 input molecules) with the amplification profiles currently in place due to PCR plateau. The dynamic range for detection of amplicons in the microtiter format is between 10^7-10^9 molecules (R. Pottahil). By modifying the amplification profile we should be able to establish a linear range of detection. Competition between Watson-Crick strands may interfere with probe capture and an asymmetric amplification system may be required. Alternatively, given the efficiency and specificity of the SK462-431 system with UNG pretreatment at 50°C, we may be able to quantitate with fluorescence alone. Stephen Scharf and Steve Will recommended using flouorescent tagged primers (ROX or JOE). ABI claims attamole sensitivity with both labels.

E) Standards.

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Project No._____
70 Book No._____ TITLE _____

1. Internal standard to monitor sample recovery. We need to first determine whether intracellular genomic RNA, viral particulate RNA, or mRNA should be targeted. For intracellular genomic and mRNA, we might consider co-amplification of a 'housekeeping' gene. For viral particles, perhaps we could spike in an exogenous, non-human virus such as MuLV. This virus would have inserted into a non-functional part of its genome, the SK462 and SK431 primer binding sites and a modified internal HIV sequence (see below).

   Potential pitfalls with using MuLV:

   a. Maintaining a stock of intact viruses.

   b. Quantitation of viral particles as there will be defective viruses and empty capsids

   c. Amplification of MuLV may not necessarily reflect condition of HIV particles in the specimen.

2. Internal standard to follow RT and PCR efficiencies. If MuLV or other "mutated" virions are not used, RNA transcripts containing the same primer binding sites but with a modified internal sequence, can be spiked into the reactions. The internal sequence should be sufficiently different so that it can be differentiated from the true target. Several approaches can be taken. These include, a) replacement of the SK102 probe region with "junk" sequences; b) complete scrambling of the internal sequence; or c) Alice Wang's HIV plasmid which spans the SK145-39 region and contains a single point mutation leading to creation of a new restriction endonuclease site within the SK38-39 region. Some of the potential disadvantages in using Alice's control are: 1) restriction endonuclease cleavage is required to differentiate between control and true

Witnessed & Understood by me, | Date | Invented by | Date
Recorded by

Project No._____

TITLE_____ Book No._____ 71

target; 2) heterodimers that form can abolish restriction sites (very likely especially at high input number); 3) potential mutation in the true target that generates same restriction endonuclease site (much less likely).

STRATEGY:

Development of this assay will have to occur as a multi-step process, some of which can occur concurrently.

1. Given the uncertainties of using MuLV as control, we will construct a randomized clone of the region flanked by SK462-431 for use as an internal standard. The SK462-431 region can be depicted as 5 separate regions: A-E. Region A=SK462, B=region between A and SK102; C=SK102; D=region between SK102 and SK431; and E=SK431. Our strategy is to retain the primer binding sites A and E, but shuffle regions B, C, and D each separately and then combine, rather than shuffle the internal sequences altogether. The intent is to maintain the location, sequence composition, and Tm of the SK102 probe region.

2. The shuffled sequences will be generated by ligation and repair of two long oligonucleotides that overlap by 6 bases of complementarity at the 3' terminus. The first oligonucleotide contains a *Pst*I linker, the SK462 region, and the first half of the shuffle sequence. The second oligonucleotide contains a *Bam*HI linker sequence, the SK431 region, and the complement of the second half of the shuffle sequence. After annealing and subsequent repair with Klenow, the fragment will be digested with *Pst*I and *Bam*HI and cloned into a transcription vector, pSP64 (with poly A).

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Witnessed & Understood by me,	Date	Invented by	Date	
		Recorded by		

Project No.
Book No.
TITLE

72

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3. After cloning and isolation of plasmid DNA, the DNA will be linearized and transcribed *in vitro* with SP6 RNA polymerase. To remove residual DNA, the RNA will be passed through an oligo dT column and further digested with RNAse free DNAse.

4. For quantitation, various amounts of RNA will be spiked into the extracted samples. Control and sample RNA will be reversed transcribed with either *Tth* DNA polymerase or MuLV RT. The decision on whether *Tth* DNA polymeraseor MuLV RT will be used depends on *Tth's* ability to incorporate dUMP in the one-step reaction and on the relative importance *Tth* vs carryover prevention. Single step RT/PCR that incorporates dUTP/UNG can be performed with MuLV and *Taq* DNA polymerase but without the benefit of RT at elevated temperature. The internal standard will serve as control for reverse transcription as well as PCR.

5. Attempts will be made to identify reaction conditions which will allow preferential amplification of RNA over DNA. For example, given the relatively low melting temperature of our amplicons (75°C for SK38-39 and 86°C for SK462-431) over complex DNA, we may be able to modify the denaturation profiles to bias amplification of the amplicon. Since RNA-DNA hybrids are more stable than DNA-DNA hybrids, one potential problem is identifying denaturation conditions which will selectively allow denaturation of the transcribed cDNA from RNA while keeping the duplex DNA intact.

This potential problem can be minimized in at least three ways. One, which we already have in place for HIV, is to design systems where the amplicons have lower melting temperatures. Since the downstream primer will be used to reverse

No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by

TITLE

transcribe from RNA, the "business" end of this potentially long transcript should still have a lower melting temperature which, hopefully will be "breathing" sufficiently to allow the second primer to anneal and extend in the first cycle of PCR at a lower than standard denaturation temperature. Second, RNAse H can be added to degrade the RNA prior to PCR. Finally, modified bases that decrease in this duplex can also skew the reaction.

The HIVBX2 RNA transcripts generated by A. Wang's group and the HIVZ6 plasmid DNA generated by S-Y Chang, provides an attractive model system for evaluating RNA vs DNA amplifications. Minor sequence variations between these two viral variants has enabled us to design type specific probes that will further facilitate analysis.

6. A quantitative detection system that has a broad dynamic range needs to be developed. We will begin by evaluating amplifications with ROX and JOE (fluorescent) labeled primers and assess signal as a function of copy and cycle number. Products will be initially analyzed on ABI's gene scanner. Samples will be amplified with dUTP and reactions pretreated with UNG at 50°C for 2 minutes. If the results are promising, the assay can be converted to a microtiter plate capture format. In this format, the products can be analyzed on two different plates, each coated with either the shuffled SK102 (for the internal control) or with SK102. Alternatively, unlabeled primers can be used and the products captured with ROX (for internal control) and Joe (for true target) labeled probes. Only SK431 which is complementary to SK102 will be labeled. Several investigators have

Witnessed & Understood by me, Date | Invented by | Date | Recorded by

Project No.
Book No. TITLE

74

From Page No.

reported increased signal detection when both primers were labeled -presumably due to network structures. A single-labeled primer may provide more accurate quantitation.

In this strategy, there are no internal controls to monitor sample recovery during the extraction procedure. In addition, while we will attempt to preferentially amplify RNA over DNA, the presence of any single-stranded DNA in the sample may compromise interpretation of the results.

To Page No.

Witnessed & Understood by me. | Date | Invented by | Recorded by | Date

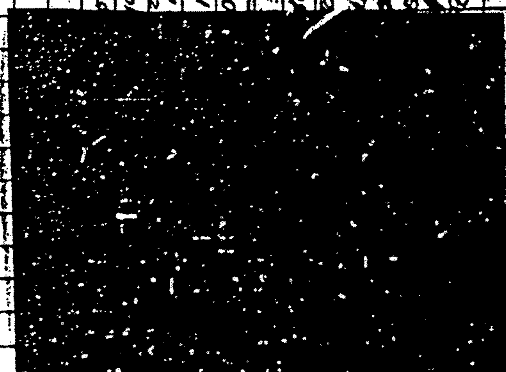
Project No.

TITLE Amplification c Joe + Rox-labeled primers Book No. 75

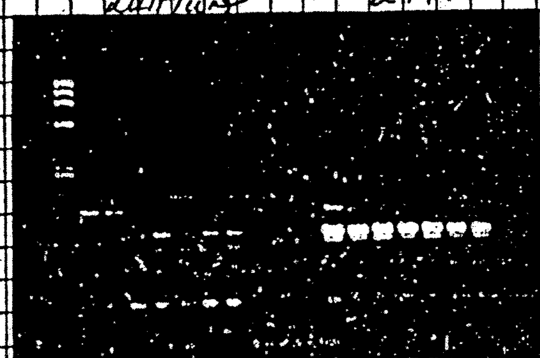
From Page No. Start

Nancy repeated ~~[illegible]~~ amplifications with JOE labeled primers + confirmed my earlier observation that JOE primers were less efficient. Repeated expt here on QC4 with dUTP/ung (Exp). Also ~~compared~~ amplified c̄ ~~[illegible]~~ Rox (Clue) labeled primers, + compared dUTP/ung vs dTTP.

Unlabeled JOE Rox labeled dUTP/ung dTTP.



JOE labeled



To Page No.

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