NATIONAL INSTITUTES OF HEALTH

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IDENTIFYING OPTIMAL METHODS FOR
CLINICAL QUANTITATIVE FLOW CYTOMETRY

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FRIDAY, APRIL 11, 2003

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The meeting was convened in the Hunter Woods Room of the Hyatt Regency Hotel, 1800 Presidents Street, Reston, Virginia, at 8:00 a.m., DR. GERALD MARTI presiding.

PRESENT:

RAUL BRAYLAN
KENNETH COLE
JEAN-LUC D'HAUTCOURT
BRUCE DAVIS
KEN DAVIS
RANDY FISCHER
ROBERT HOFFMAN
BURT HOUTZ
ERIC HSI
LANCE HULTIN
LARRY LAMB
RODICA LENKEI
GERALD MARTI
PHIL McCOY
KATHY MUIRHEAD
ALBERTO ORFAO
NORMAN PURVIS
JORGE QUINTANA
LEA REICH
ABRAHAM SCHWARTZ
HOWARD SHAPIRO
MARY ALICE STETLER-STEVENS
KAREN TAMUL
KATHLEEN THOMPSON-DAVIS
ROBERT VOGT
JEFFREY WANG
JAMES WOODS
YU-ZHONG ZHANG

A-G-E-N-D-A

AGENDA ITEM

Session III: Data Analysis, Quality Assessment, Reporting Results
- QC for Instruments
- QC for Reagents
- QC for Sample Prep
- Standardizing Data Analysis / Path Interpretation
- Reporting QFCM Results
- Identifying and Reporting Sample-Specific Anomalies
- Workgroup Assignments and Breakouts
  - QA / Standardizing Data Analysis
  - Reporting Results

Session IV: Summary
- Plans for Experiments to be performed Prior to Follow-Up Meeting
- Sample/Calibrator Exchanges
- Reagent Development (companies)
- Clinical Trials

P-R-O-C-E-E-D-I-N-G-S

(8:28 a.m.)

DR. MARTI: Good morning. Good morning, everyone. Welcome to the second day of this conference to try and define what the problems are and some appropriate solutions for clinical quantitative flow cytometry or quantitative flow cytometry in a clinical setting.

We thought that in order to start the session today, we would start out with compensation, have part of the discussion about compensation.

DR. VOGT: As opposed to decompensation?

DR. MARTI: As opposed to decompensation. Recently we had a couple of experiments in our lab, one involving the CE79D. As you came acutely aware that this particular reagent -- I don't remember whose company it was or was. I don't remember what the fluorochrome is. We realized that the nonspecific background was pretty high, that the t-cells were staining more than we were used to. And it reminded us that we hadn't titered that reagent. When we titered it, it behaved very nicely.

The second thing in this series of experiments, as we were moving from four-color to six-color, we carried out an experiment on the LSR2. We dutifully were doing a comparison between manual compensation and automated compensation. We constructed for this experiment all of our single tube controls using CD3: CD3 FITC, CD3 PE, CD3 PE 5.5, APC, the six reagents that we were using.

And when we did it, we ran those. And we were comparing the six-color to the four-color. We essentially had such terrible overcompensation on the FITC PE that we actually didn't know whether we had made a mistake in the sense that the fluorescence intensity of our controls was the same or lower than the test panel or that conceivably that it might be above or failure in the software. That is an easily testable problem, and we are looking into it hopefully this week.

However, in the process of this, it made me think once again about compensation controls. When we were discussing this with Steve Perfetto in Mario Roederer's lab, he mentioned to us that he had received some microbeads from BD.

I don't know what division, whether it was the West Coast or Europe or somewhere in this

BD operation. We tracked down these microbeads that contained I think goat anti-mouse kappa. And, as you know, most murine monoclonals are kappa, kappa Verity. And we thought, "Gosh, that's a wonderful idea to make a compensation control."

And we thought that, you know, we have had a little experience with antibody capture beads. So the first word out of my mouth was, "Well, how long did you incubate?" Oh, 15-20 minutes. And what else did you do? Did you keep buying them? Well, no. We fixed them.

And, you know, all the time we used the antibody capture beads, I don't think it ever occurred to us to fix them. We looked at them over an hour, four hours, one day, one week.

So we got a sample of these microbeads and stained them. When we stained the first set of these microbeads, we were kind of surprised to see that with the FITC fluorochrome PE and PE-Cy7 that it wasn't quite the homogeneous peak that we thought we were going to get.

We didn't know quite what this represented. I mean, is the antibody coming off of the beads? Apparently you have to be very careful with some of these tandem conjugates. They

actually form end-to-end dimers. In fact, you know, there is a very ancient procedure in immunology where when you take your reagent out of the refrigerator, you are supposed to take an aliquot of it and walk over to the microcentrifuge and do three minutes or something.

In this area, these colors, when you get up above four or six, I think we are going to have to really pay attention to that.

DR. LENKEI: The picture is still very bad. It's not a clean picture.

DR. MARTI: I couldn't agree with you more. So we got a fresh sample. That is four or six of the eight, four of the six. I overlapped it this morning because we just did this experiment.

Now I was similarly impressed with these antibody-coated beads because the first thing was they looked awfully bright to me, which suggested that that might guarantee more often that the brighter your antibody in your control, compensation control, the better chance you had of not having it be the same level of fluorescence intensity or, God forbid, less.

I think if it's less and you put it through the automated algorithms, and you are going

to be in big trouble, big trouble.

The second thing -- and I haven't had a chance to look at the statistics. It sure seems like an awfully tight CV to me for an antibody-captured bead.

The difference between this sample and the previous sample was two months in terms of sitting in the refrigerator. So I suspect that there may be some shelf life in and out of the refrigerator, et cetera.

DR. QUINTANA: Can you do it the same way, this one and the previous one?

DR. MARTI: Well, you tell me. I mean --

DR. MUIRHEAD: There is or isn't.

DR. MARTI: You can take your pick. I mean, we thought that was the most representative of what was there. I think there is a lot more aggregation in the over-sample compared to this.

DR. SCHWARTZ: Actually not. The bigger peak is a doublet peak.

DR. MARTI: Well, nice that you should point that out, but walk your eye up from the lower one to the top one. That doublet has disappeared. And I would say that the side scatter signal has

increased slightly.

What's the only difference between these three or, actually, between these two and that one is that this required a different laser excitation. So I think some of this scatter difference is wavelength-dependent or it's a different optical pathway of the second laser through the optics.

Howard?

DR. SHAPIRO: What's that little peak below the main peak in the PECy7? That's what I'm saying. The problem is basically I think what you are looking at there is that the gating is not the same for that because you can see if you look, there is a lower side scatter peak to the left of your gating region in there.

And my impression would be that those would be the singlets and that you are basically discriminating orientations of doublets.

DR. MARTI: You think that all represents doublets?

DR. SHAPIRO: Yes, because what do you have, sperm beads, in there? I mean, how do you get --

DR. SCHWARTZ: Have you looked at it in

the microscope?

DR. MARTI: Not yet. Well, not so much shame on me. It's that we didn't have time to set up to do the -- I do want to photograph these because we've always photomicrographed beads that we've used, and it's always very enlightening to see poikilocytosis and variation of hypochromia.

DR. SHAPIRO: If it will break streptococcus and staphylococcus, we may have more than a vested interest in staying together of the beads.

DR. MARTI: We just took these out of the bead bottle. You drip them out into the tube and "stain them like cells." I only brought these. We have hardly had time to think about this experiment. But I thought -- and perhaps you will correct me -- that it might be a good way to introduce the topic of compensation.

DR. SCHWARTZ: How are you going to compensate with one population?

DR. MARTI: Well, that's a good question. The fact is the Purists chided us -- and I haven't shown it here to you -- chided us for even thinking about mixing these individual ones together as a task at the end.

DR. SCHWARTZ: You cannot. It's true.

DR. MARTI: Why?

DR. SCHWARTZ: Because you have overlaps in things, but you should have two populations if intensities could be able to --

DR. PURVIS: You've got to know what the background of the bead is.

DR. SCHWARTZ: No, not background. A second population of the same label fluorochrome. And if you have two of them that look like they're compensated --

DR. HOFFMAN: This is not in the same tube for this software.

DR. SCHWARTZ: It doesn't have to be, but it won't hurt it because there is no mixing of fluorochromes. There are just two separate populations at different intensities.

DR. SHAPIRO: If you actually run two or more of those populations, you could basically do a regression line so you get a much more accurate measure of the contribution. You basically need to define a single number, but you probably can define it somewhat more precisely if you have two intensity levels or more intensity levels.

DR. SCHWARTZ: No, that is not the way this --

DR. SHAPIRO: Yes, I understand that, but how do you solve the problem overall? I mean, the software that we were playing with years ago to do this, we actually did regression lines and --

DR. HOFFMAN: This doesn't improve the matrix, the actual compensation. Basically you measure the spillover of every fluorochrome or --

DR. SCHWARTZ: Right. But the space from one population is more comfortable if it's on two or more, I would say.

DR. HOFFMAN: Well, it takes software to do that. It's a measure of each reference of each fluorochrome with all of the other fluorochromes in the map.

DR. MARTI: I don't think it's --

DR. SHAPIRO: Actually, for purposes of compensation, it doesn't matter what you deem are doublets because what you really want to see there is to what extent the fluorescence from the individual is bleeding to the other.

And, yes, if you want a single value, you probably are better off having your compensation sample as bright as you can.

DR. SCHWARTZ: As a matter of fact, put a gate around both of them. You do have your two populations. You can solve it.

DR. MARTI: So, again, I brought this map not to be definitive or anything. It's just I grew used to having a mixture of MESM, FITC, PE beads. And I like the opportunity to do compensation across the whole scale. So it wouldn't be impossible to use this system, but I do think, as Bob pointed out, that the algorithm requires or works with just a single peak. And with that peak being brighter than your test reagent, you should be okay.

Now, I understand there are further discussions about which type of algorithm is being used. But obviously since we can't discriminate singlets or doublets, we're not ready to look at differences between algorithms.

One would hope if that were necessary -- and, actually, it has become necessary because, as I said, our first experiment was so disappointing nothing turned out right except the FITC PE.

So, anyway, what kind of controls if you were running your lab for compensation would

you use or recommend? If you were doing a CLIA inspection and you walked in, what would you want to see in the SOP?

DR. HOUTZ: Biologicals and set of beads.

DR. MARTI: Biologicals?

DR. HOUTZ: Biologicals and set of beads, yes. The spectral difference between beads themselves is pretty low.

DR. QUINTANA: We have a fluorescence issue also with the FITC.

DR. HOUTZ: A lot of fluorescence differences.

DR. QUINTANA: There is a little bit of difference on the FITC, not on the longer wavelengths but on the FITCs, you see some difference between the beads themselves. It depends on the prep method, too.

DR. HOFFMAN: I think you should be able to set up the compensation with beads as well as a test method using the full matrix. But what is really important is that, especially for any tandem fluorescence, it be the same lot of the reagent that you're going to be using in the sample.

Especially in the PE, I am not sure how it could affect the FITC too much, if you compensated with a different antibody or a different lot of antibody for any of the tandems, PE tandems, that could affect your spillover matrix in every --

DR. MARTI: Every dimension.

DR. QUINTANA: But we are looking at the issue with the tandems because there is a type. It depends on the anti-bacterium specter when you prepare the tandem dye in terms of the bleed-over. You have to have a very tight specification.

And we did a study looking at full matrix comp looking at different tandem degradations. And we find still the full matrix system we have been using gives us a little wiggle room that it's about a five percent difference in the bleed-over that really has very little effect on the compensation at all in terms of moving the populations.

I agree with Bob. You can use the exact dye lot. It's the same. What happens is over time, some of the tandem dye lots do degrade from time zero to, let's say, eight or nine months. So it's one of the things that you have to stay on

top of, especially even mixing manufacturers' tandem dyes. You really can't because there are different specifications.

DR. MARTI: I think the bottom line is -- and I think you folks said it more eloquently than I probably will -- whatever you stain yourself with and whether you use cells as a compensation control or bead, it has to be stained not only with the same antibody but with the same antibody, particularly tandem conjugate.

If you have the same lot of monoclonal antibody but the conjugate was prepared at two different times and, therefore, two different sublots, we got burned in a lot of these things in a big hurry. It didn't take long. It just jumped out at us and kind of beat us up.

DR. FISCHER: So, Bob, I have a question for you. You say you prefer beads because of the automated software.

DR. HOFFMAN: I didn't say I preferred beads, but --

DR. FISCHER: Well, that's what I heard. And so I guess that's what I --

DR. MARTI: No. He said there would be no reason why beads shouldn't work.

DR. HOFFMAN: And they do. The software works about the same as --

DR. FISCHER: My question is, how are we in the clinical labs running the new software with the compensation?

DR. HOFFMAN: They're doing one or two-color. And they have the option of using the automated, fully automated. I sure wouldn't do it. Doing two-color compensation actually is pretty easy.

DR. FISCHER: What's the software that does that automatically?

DR. HOUTZ: Fax Comp.

DR. FISCHER: Well, Fax Comp works on calibrated beads. My understanding is it doesn't work on NESF beads.

DR. LENKEI: No. In the preliminary means, still there are some variations among colorblind beads.

DR. FISCHER: It's close, but it's still not best. From everything you just said, it's got to be the right dioxin. It's got to be the right things for you to --

DR. SCHWARTZ: It has to be the right intensities for their compensation.

DR. FISCHER: Right. That's what I mean. So --

DR. HOFFMAN: Less right, correct intensities. But with calibrated beads, they don't have to be exactly the same intensity as antibody clonal beads. We tried putting the correction factor in there that will get you close. And that actually is I think the right bead.

DR. FISCHER: So to come back to the question of who in here will have the automated software, I have seen them on the SR2. It looks really nice, but my calendar doesn't run that.

So we have to go back to the question Jerry posed at the beginning of how do we do our compensations when we're doing them essentially by eye. You're still adjusting it with your eye and looking at the numbers a lot of times --

DR. MARTI: Randy, you can collect uncompensated data on the fax calendar and then analyze it with flowcharts. I don't know about Winlist. I assume you can. I would assume you can.

I think that the whole idea of collecting uncompensated list mode data in the clinical lab -- and people correct me if I am

wrong. There is nothing wrong with doing that, and I think there is some experimental data to support that that is a pretty good idea.

I think that they have to understand that the clinical budget is going to go up because all of those reagents that are being used will have to be run as single color controls.

DR. PURVIS: Not only that. Also, you have multiple instruments, and you're doing samples. You've got to know which instrument it came off of. I mean, you've got to look at the serial number embedded each time that you pull up a file.

DR. FISCHER: Let's talk to someone like Mary Alice who runs a clinical lab. Are you going to want your people to be taking all of their data uncompensated onto the FlowJo and doing all of this --

DR. STETLER-STEVENS: We're considering it. I've wanted to do it for quite a while. We haven't been able to move the force to do it because, first of all, they're saying, "Oh, God, no."

Another thing, what about the Coulter's FC500? They have a different setup for

compensation. We bought one, and it seems to work pretty nicely. I'd like to hear about --

DR. QUINTANA: The FC500 can do compensation that points at data. So all the compensation assistance is done in linear. It's not done on log. It's displayed with a lot of echoes with a front log look-up table.

The FC500 has this ability to do post-acquisition compensation, where you can actually take all of your data uncompensated, you can run your compensation setup any time during the day as long as it is the same setting, and then you can apply that compensation to the linear data.

It also gives you the capability to reanalyze the data to manually compensate it. It's not something we recommend, but using the compensation setup, it allows you to go back. An application for that would be to say you've looked at it and you have a concern.

We use CD45 single colors along with a control cell as setup for the compensation. What we found is that with the control cells and running those control cells, it gets us a lot closer of a fluorescence measure to at least the samples that we have run through our light systems. It's as

close as it is of any of the other systems that we have looked at.

We can go back and actually set up setup panels and go back. You can reanalyze the data by rerunning a different lot of CD45, let's say one of the tandem dyes. If there is a different manufacturer, you can actually re-create a panel and reanalyze the data and apply the compensation to it. That offers a lot of flexibility, but it still keeps the operators from having to do any manual compensation because you will apply the standard auto-setup panel to run the data.

DR. LAMB: You know, I've got a real problem with post-acquisition compensation, especially in a clinical setting, because you can make things what you want them to be. What's the difference?

I mean, you get your instrument setup parameters that you use. If you're doing a leukemia, for instance, you set up your instrument according to manufacturer's instructions, which basically you are tied to if you are going to have the same thing from day to day to day and pass your CAT inspection.

Maybe I am living in the past, but "It is what it is," to quote Popeye. This is a problem I had with people at one point in time who bought a Becton Dickinson instrument and didn't like to buy PerCP. So they bought the other manufacturer, Coulter's or Caltag or somebody like that, or FACSCalibur. And then they ran all of their leukemias uncompensated and came back to Winlist and fixed it later that day.

DR. MARTI: Okay. He had his hand up for a while.

DR. SCHWARTZ: This is ten years ago. I remember one of the same concerns about doing this is should we have list mode files at all. You should just get what it is is what it is. And we are going to take that histogram, and that is all we need.

And now I don't think there is anybody here who does approach it like that.

DR. QUINTANA: The post-acquisition color comp is not something that we would intend for the clinical labs.

DR. SCHWARTZ: But it can be --

DR. QUINTANA: When you have the clinical setup software, it is going to run through

the algorithms and do what it needs to do.

DR. STETLER-STEVENS: Although there are situations when you have --

DR. SCHWARTZ: You want to tweak.

DR. STETLER-STEVENS: -- a very small specimen that are patients who have been treated and you have weird autofluorescence and you have funny findings and the compensation comes out bad.

But we have like 10,000 cells in the whole system. We can't adjust the compensation. You can, but if you run it, it's gone. It's done with.

And sometimes it would be nice -- well, I compensate with my eyes on that point. I go, "All right. I know what that is. And I figure it out." But sometimes I think there would be a place where post-acquisitions could go in and toggle their little --

DR. WOOD: I think that there needs to be a clarification here, and that is what it is that we are storing. We want to store data. We don't want to store information. Once you have compensated it, you are now storing information. And you are imparting a bias field already. You want to store the most unbiased version of what is

coming off the flow cytometer.

So ideally what you would like to have is raw linear data that hasn't even been transformed by any type of a log display, but not all instruments have that.

It's not unlike what was available with Electric Desk. I don't know if people remember that from way back where you could run a virtual flow cytometer at your desk that data was collected and then you could go back and rerun the experiment, adjust settings, and so forth, and deal with the fact that your operator didn't necessarily know how to set all of the fine adjustments. What you did there is you stored the data, not information.

So I think that we need to be aware of that and actually break away from the old paradigm of when you store histograms. The histogram and display are one and the same.

What we need to be looking at is there is data that is stored on the computer. And then there is a display that can be generated from the data, but the display is not the actual data. It's a way for you to visualize it on your standard. And the two of them are separate.

DR. FISCHER: It's my understanding -- and I can't speak to the Coulter's because I have not -- it sounds to me like they are also now saving it as uncompensated data, but I'm not positive on that.

I know that certainly on the new instrument, all of our data saved uncompensated. It's just the way they designed the storage. And then you come back and actually do the compensations post-acquisition because the data now is all saved.

I can't speak as to whether it's there. I wish Gordon were here so he could speak to that. Phil, you might know. I don't know.

DR. McCOY: I think it's log. Actually, you can store both, store in one list mode panel both compensated and uncompensated.

DR. QUINTANA: The FC500 does the same thing. It saves a 10-bit compensated and a 20-bit length of 20-bit linear data. You have both models.

DR. FISCHER: And I know that mine does the same thing. The Calibur does both. I'm assuming at some point in time they may decide to switch that to a clinical instrument. I am not

sure they are going to want to do that. The research instruments obviously are all going to be that way because the research --

DR. HOFFMAN: Any future instruments are going to be that way. Any future instruments are going to have digital high-resolution linear data on that.

DR. TAMUL: But that's the future. I think that we need to get back to the real world here for a second. The majority of clinical labs that are going to try to do this are not going to use FlowJo. It's too complex to carry out in three to five years.

But everyone in here is running high-powered labs with really serious effects. The rest of the world out there is not going to quite be so sophisticated and able to do it.

The only other thing I wanted to mention was that perhaps a definition or a little bit of change in semantics might help. When I was in the lab, what we used to do is called instrument setup or calibration with beads and instrument setup, then compensation adjustments and any other adjustments with biological control as optimizing for the samples *in vivo*. That might help clarify.

DR. STETLER-STEVENS: I have to say if you're going to compensate online, you need to have a really experienced tech doing it and that --

DR. TAMUL: That CID up there.

DR. STETLER-STEVENS: Yes. Out in the community, when you have people who don't know what they are doing, compensate --

DR. QUINTANA: The software system has the --

DR. STETLER-STEVENS: You have to go with something that is automatic.

DR. HOUTZ: And you can do it automatic after acquisition, post-acquisition to be automatic.

DR. MARTI: Howard and then Bruce.

DR. SHAPIRO: I apologize for delaying this, but I don't see any way around it. First of all, when we say "post-acquisition compensation," we're really covering a moment to things because of the different ways in which different instruments process the data.

If we are talking about the instruments that are in the majority of clinical labs -- and, you know, there is a dichotomy here. We have got Scans or FACSCaliburs, which are probably the most

recent clinical version of the old style instrument.

That is, the Calibur has got, what, 10-bit A&D converters? So basically you've got 10-bit data. You have got 10-bit linear data. And if you want to do post-acquisition compensation to that, you have to convert the log to linear and back. And you lose data. The thing is kind of granular. To make the data look halfway decent when you display them, you've got to diddle and add random numbers and things like that.

So, in essence, on the other hand, if you were doing a three-color measurement on a Scan or Calibur, you could certainly compensate a two-color measurement. A lot of people can compensate a three-color measurement.

Four colors is dicey. I don't think anybody can really compensate four colors effectively by eye. When you go to more colors, you can simply forget it.

Now, if you look at the major competitor of the Calibur, which is the XL, Coulter XL, Coulter XL has done away with the log amps. It is collecting 20-bit linear data, which until recently was not accessible to the users. The

newest software of Coulter gets that data out there. And the FC500 has got the digital software.

So when you do that, when you collect 20-bit linear data, there is no log amplifier involved. You have got the raw data. So you can shift from linear log and back. You don't lose any precision in the measurement. And it's perfectly feasible to do post-acquisition compensation.

Remember, if you are doing more than four colors, you basically have to do matrix compensation if you really want to compensate the data, certainly if you are doing four or more. We could argue about three.

First of all, what we are talking about here, we are talking about doing quantitative, clinical quantitative, flow cytometry. It is not going to be possible to do that, certainly not on three-color instruments, probably on our four-color instruments, out there in labs in community institutions. it's just not going to happen.

We now are all pretty much used to doing four colors for CD4, CD8. We know that is an improvement. And yes, you can still get by the guidelines and do it with a two-color instrument. Some people are doing that.

But if you are trying to be on the cutting edge, you have got to be doing four colors. My guess is that to do what we need to do with the gating parameters that are involved, we are talking more than four colors. And almost all the instruments that are doing that are going to be doing digital processing.

Okay. I didn't mention Cytomation. Cytomation has got log amplifiers, but they convert the log data to 16-bit data, which allows them to move it back and forth between linear and log without screwing it up too much. And they also can compensate for their log amp response. And BD with the Vivo Electronics, again, it's got a high-resolution linear data.

So basically you can always store the raw data in a form in which you can shift back and forth between linear and log and which you can shift back and forth between compensated and uncompensated without degrading the number that you have. That is the important point.

By the time we actually get through -- when we start doing this, if we set up a protocol now, the labs that did it would all have the wherewithal to do it the right way, even with the

high-resolution linear data.

And it wouldn't really matter. It doesn't matter if you run the compensation samples first and you collect the data compensated. You still have access to the uncompensated data. So whether you run pre or post-acquisition compensation is not going to make that much difference.

DR. DAVIS: My only point is I think we haven't asked the basic question, if we're really going to do quantitative data, should we do any compensation?

I mean, my data with what we do is if you compensate, there is no way you are going to get the same value from different instruments. And if the whole idea is to do quantitation where one lab gets the same answer as another, you should at least start with a base protocol where you understand the effect of compensation between instruments and all of that because if somebody --

DR. SHAPIRO: I agree with you that if the channel that you use for your quantitative measurement is one in which you don't expect any cross-talk, then a quantitative measurement is going to be cleaner.

But that is a separate issue from the fact that in the context in which we are talking about doing quantitative measurements, we are almost certainly going to have to use enough gating parameters that we have got to compensate to get the population on which you are going to do the quantitative measurement.

DR. MARTI: Bob, then Jim.

DR. HOFFMAN: First of all, there are not that many. With the existing instruments, your situation is limited to what accommodations of fluorochromes you can use for specifically reliable quantitation.

So like with the BD Systems -- people are going to ask. They're going to say like "Who's this?" You set up a system that is robust and reliable. We would use a PE. If we are going to quantitate with PE, we would use PerCP or PerQM. ABC doesn't have any. So you use accommodations other than FITC that don't have any cost docked in the PE.

The only two fluorochromes that Billy talked about, at least in the near future, quantitating, are FITC and PE. Those actually are the two that you can pretty reliably compensate for

on any system.

DR. LENKEI: But you have to compensate.

DR. HOFFMAN: So you're just more limited to what fluorochromes can be compensated on and what combinations of fluorochromes you would use in multi colors, for instance.

DR. MARTI: Jim?

DR. WOOD: Well, one of the things I just wanted to bring up in terms of doing your post-acquisition compensation; that is, some instruments only have what I have called subtractive compensation.

With that type of compensation, the only thing you can do is two colors. Those two colors are sought exactly by subtractive compensation. Three-color cannot be. Four-color can be if you have, for example, colors 1 and 2 interacting and colors 3 and 4 interacting, but you can't have 2 and 3 interacting.

So taking that into mind, then some of the older instruments really can't do compensation at all beyond two colors. So you have to do post-acquisition unless you choose the colors real carefully, in which case, say, for example, if you

are doing three colors, the third color didn't need compensation to begin with.

Then in terms of choosing colors, one of the things to keep in mind is trying to minimize the cross-terms that exist. If, for example, you can keep one and two so that they are only interacting, three and four interacting, you don't have much two and three, then the compensation matrix will be very simple. And the instrument or the map won't be working quite as hard trying to compensate.

DR. MARTI: Kathy?

DR. MUIRHEAD: To get back to Jerry's original question, which is what do we need to worry about, I think the answer is if you under-compensated in your sample, it probably doesn't matter. If you, without realizing, over-compensated, you're going to lose data, and you're not going to know it, you're not going to --

DR. FISCHER: I've actually seen that stuff come through from other laboratories. They've sent it through me and asked and said, "Well, you know, we are having a problem. We know you do a lot of flow. Could you tell us what is wrong with this thing?" And I look at them and

say, "It's compensated. Where is your data?"

My Calibur saves all my data compensated. It's gone, which is why I think they went to the uncompensated because now if you screw it up when you do the printout, you can just simply go back --

DR. MUIRHEAD: It's arguably more time, especially if you have got a system that enables --

DR. SCHWARTZ: What takes more time, doing that or another sample?

DR. MUIRHEAD: Not being able to get the sample.

DR. FISCHER: The sample is not available.

DR. SCHWARTZ: That is even worse.

DR. FISCHER: Compensated, you have a chance of going back and doing things with the data.

DR. SCHWARTZ: Yes.

DR. LENKEI: Compensation can be dangerous because then you don't see. If you gate on subsets of cells and aren't interested in double positive cells, then if you type the compensation, I don't see the double positive.

If you type the compensation, then I

see application of double positive cells. So it depends from the clinical point of view. If we send a Branch Lodicom to measure and the others forget, then it is important. Otherwise, probably to look at one pair and one a cell, there's a lot being compensated, but it's not because it's not effective.

But we studied that, and I checked both sets. I think we have a very good compensation, even checking on the negatives.

DR. HOFFMAN: The Calibur doesn't do full-matrix compensation, but PerPC or PerPC10, which doesn't have any spillover back into the FL3, what we don't compensate is -- so it's really the FL3 channel that will never be perfectly compensated by most --

DR. LENKEI: And the problem is that you are inferencing Calibur. And then if you use Calibur as compensation, sometimes you can get into trouble. It happens.

So from this point of view, if you have CD3 concomitantly with Calibur, then it's no risk. Then you know how Calibur is looking, but you see still that it's a good compensation.

DR. SCHWARTZ: Let me ask a question

that is kind of rhetorical. When you compensate, do new populations appear or what is the uncompensated is still the compensated, but it looks pretty because it is --

DR. SHAPIRO: The only pseudo new populations are the ones on the bottom of the scale. There is some work being done to deal with that. I don't know. Maybe this would be an appropriate time to show that slide.

DR. SCHWARTZ: I mean, the compensation doesn't make things appear that weren't there before. So other than making it look pretty, why bother? You can do gates.

DR. HOFFMAN: That's actually not quite true. I mean, the example I used of being very densely stained, double stained, CD45, FITC, and a very dim DE. In a normal dot plot, in that example, you have to resolve those uncompensated.

DR. SCHWARTZ: So things do appear when they're compensated?

DR. HOFFMAN: Resolution of the --

DR. SHAPIRO: No, they don't really.

DR. MUIRHEAD: Well, I think he's asking, do they become more discrete?

DR. SCHWARTZ: Could you --

DR. SHAPIRO: The mathematical distance between those in the linear space --

DR. SCHWARTZ: But could you find the population so you can do quantitation on it? That is the question.

DR. FISCHER: The answer is no sometimes.

DR. MUIRHEAD: But if you I think don't --

DR. WOOD: That's not really true.

DR. LENKEI: Yes, you can, but it depends on the --

DR. MUIRHEAD: You can.

DR. WOOD: If it's not distinguishable when it's uncompensated, it will not become distinguishable.

DR. FISCHER: The problem is if you have the bleed-over, how can you tell if bleed-over if you're looking at it from the quantitation, until we get to the point where we can do two-color quantitation --

DR. WOOD: Compensation is a crutch. Okay? Once you understand what the problem is and understand what the clusters are, then you can actually solve the problem on a routine basis with

uncompensated data.

DR. SHAPIRO: Yes, but compensation isn't a crutch for a human in the sense that when we are working, we start working. The data that we collect are collected in what I will call a color space. So just you use a three-color thing.

We're basically using green fluorescence and yellow fluorescence and orange fluorescence. What we want to get from the green fluorescence -- or let's say green, yellow, and red. What we want to get from green, yellow, and red is fluorescein, phycoerythrin, and PerCP or PECy5 or something like that.

The green signal is mostly fluorescein, but it's got some PE and maybe a -- well, it probably doesn't have very much PerCP or PECy5. The yellow signal is mostly PE, but it's got the chain and probably maybe some of the other things. The red signal is probably going to have a little PE in it, probably not very much fluorescein.

The compensation gets us closer to the numbers that we expect to quantify the individual labels, which, in turn, is what we need to quantify the antibodies found per cell.

And so in that sense, the compensation

is not a crutch. The compensation is necessary to get us the information. Compensation was basically invented at Stanford to be able to sort cells using that kind of a gate.

DR. STETLER-STEVENS: Can I make a comment about observations on compensation that it is not perfect when you are doing it manually? I have someone really good doing compensation, and I have really intelligent people. But it's not perfect. And you have a range of fluorescence that you're looking at.

One of the cases I showed you early on was when we were looking at an antigen trying to quantitate it, when we kappa in one tube and lambda in another tube, we see differences. We see a difference when we have an extremely high level of bright staining with the light chain in the one tube and it's negative in the other.

The antigen that we're quantitating changes. It shifts. And this has to do with compensation issues. Yet, it looks well-compensated. Yet, it's enough of a shift to change the numbers. If you're not interested in quantitating, you say, "Look, it's positive." And that is your answer.

So if we're doing clinical, you know, leukemia immunophenotyping, it is perfect. But when it comes to quantitate, you see shifts in the numbers of the fluorescence. It has to do with it has to be compensation because it is another antigen that is either positive in one tube and negative in another. You see differences.

DR. SHAPIRO: I think the summary compensation is -- we were talking about English majors the other day. This is not who wrote Shakespeare. Compensation is a mathematical process. You know, there is an equation to solve, and there is a right answer. And that is the way it's done.

In terms of what we need to do if we are going to do quantitative flow cytometry, clinical or otherwise, you have to use matrix compensation for just the reason that you said. Manual compensation will give you data with which you feel comfortable, but you have no guarantee that you have the right numerical answers.

So we don't need to discuss this anymore because basically if you want to do this right, then you have to do it on an instrument which allows you to do matrix compensation,

preferably on one that keeps high-resolution digital data.

I would venture to say that the labs that pilot this are all going to have access to that equipment. And the labs that want to prove this in the clinic are going to have to acquire that equipment.

It would be very nice if the manufacturers made the software, made the use of those instruments as easy as possible, but, as Einstein said, no easy.

DR. HOFFMAN: Just a small correction. You have to do correct compensation. Okay? Like in the case of the --

DR. SHAPIRO: Okay. Yes, yes.

DR. HOFFMAN: It does not have to be matrix as long as you are correctly compensating the channel fluorescence that you are compensating.

DR. STETLER-STEVENS: Which is --

DR. HOFFMAN: But it does have to be corrected, and it can't be to whatever your taste is as to what the data should look like.

DR. LENKEI: But it just may take you to use adjust to correct for this type of high-intensities in some because you make the

compensation adjusting to one tube which you run. So it would be the same problem.

DR. SHAPIRO: No, no. There would be a right way to do it. I wouldn't worry about that. I don't think the intensity is as big an issue as --

DR. LENKEI: No because you titrate the in-tube art. Then you cannot --

DR. HOFFMAN: No. There is only one correct setting. If you don't change your PMTs and you are using the MPE, there is only one correct setting. The data may not look the way you want it to look. If they would be broad for -- I agree, but that is the way it should work.

DR. MARTI: And then I would like to try and summarize this so we can move on to the next comment.

DR. D'HAUTCOURT: I will make one more distinction. It is not only manual versus automatic compensation, but it is also hardware versus software.

In my opinion, we must agree with the finding about three-color. There is no art work possibility. So there is only one way to do multi-color. It is to do the inverted matrix.

This is only done with software.

DR. SHAPIRO: You can actually implement that matrix in hardware.

DR. D'HAUTCOURT: Yes.

DR. SHAPIRO: So yes, you're right. That's correct. Basically hardware compensation is going the way --

DR. MARTI: You know, several years ago Carlton wrote a paper on compensation and pointed out very well the application that you get in hardware if you try to do that subtraction first.

Of course, there was an exchange of letters between him and Mario over exactly what that meant. Then Mario published three papers back to back in *Cytometry* dealing with not only compensation but data exploration.

I think that you have all been in the field long enough that you must have seen publications in the *New England Journal of Medicine* and *Blood* that have lousy histograms.

When you start looking at four-color data, it is frightening. I mean, it sends shudders. I mean, there is probably only ten percent of four-color that is probably just going to be correct.

DR. SHAPIRO: The question I wonder about is clearly -- you said a journal. A good journal sends something out to maybe three referees. A paper these days, it's got gels. It's got microarrays. It's got flow. It's got this. It's got that and the other thing.

So I don't think flow is isolated. I think there is a lot of crappy array data out there and a lot of crappy gel stuff.

DR. VOGT: Yes, right.

DR. MARTI: If you think standardizing flow is great, you ought to try 18,500 spots on the microscope.

DR. VOGT: Gridlock is right. There is, by the way, an NCCLS microarray committee, which is moving quite expeditiously or they exchange e-mails regularly. I have seen them. I haven't looked at them, but we hope to have some forum for discussing that at the CCS meeting in November.

DR. MARTI: Just to see if I have interpreted or heard correctly, at some level, at perhaps three but certainly four and more, this group would recommend automated compensation inside if that's possible.

And ideally somebody made a comment that the clinical labs that want to move toward quantitation in the setting of three or four or more colors is going to have to acquire --

DR. SHAPIRO: Let's say high-resolution because, in fact, I think that if you talk about the accuracy of present methods, that the Coulter approach, which does not do digital pulse processing but which gets a 20-bit number, is probably slightly more accurate than the digital pulse processing approaches.

And as we are able to convert more bits worth of data, the distinctions will go away because ultimately full digital is better once you get enough resolution, but we don't need to argue about that because you can get high-resolution linear data.

And, actually, as I said, even with cytomation, with log amplifiers, if you digitize log data to high resolution, then you also get to the point where you can convert to linear log and back.

Ultimately what we really need is for the instruments to have the gains calibrated so you can change the gain and figure out what the new

compensation settings are going to be. That is doable, but how soon it will take to implement that, I don't know.

DR. HOFFMAN: I agree with that statement. If what you're saying is that you want to have the capability, the flexibility to quantitate on any of your fluorochromes, that is true.

In any of the existing systems, even with four colors, if you choose the right combination of fluorochromes and the right fluorochrome is quantitated on --

DR. LENKEI: Yes.

DR. HOFFMAN: I mean, you can do that perfectly well within your equipment.

DR. LENKEI: If I had been stuck, you told me that after four colors, the matrix compensation was really good. I think on my Calibur, that is the best I can do, quantitation.

DR. MARTI: The fact is that's where you can automatically start looking for bad data in the literature.

DR. LENKEI: Yes.

DR. MARTI: The PECy monitor.

DR. LENKEI: Yes. And I don't think

that any contents would be recommended for quantitation because too many subsets of data.

DR. QUINTANA: What about correct compensation? Correct compensation can be correctly set with the lower-level instruments if it's done properly and it's the right fluorochrome. Is there any other form of algorithm that has a different name matrix? Coulter is using a matrix. So that seems to be what is --

DR. QUINTANA: It's available in the XL now as well as the FC500.

DR. MARTI: Are there any other mathematical methods?

DR. HOFFMAN: The correct compensation actually covers --

DR. QUINTANA: The XL software --

DR. HOFFMAN: It does either what we call conventional --

DR. QUINTANA: Right.

DR. MARTI: Okay. On that note, I am going to conclude that --

DR. SHAPIRO: There's something I want to show related to compensation. And it's also related to log scales. I think it's important. So it's in a late-breaking news section of the fourth

edition.

DR. VOGT: Does compensation work if you don't have the cables hooked up?

DR. SHAPIRO: I think there are some people in my lab who think so.

DR. VOGT: Maybe it works better.

DR. SHAPIRO: Here it is.

DR. VOGT: Good.

DR. SHAPIRO: One of the problems that you run into, one of the problems that you run into, with high-resolution digital data or not quite high enough resolution digital data, at the bottom end of the log scale when you do a conversion is that you run into what we call the picket fence, in fact. And they just start to get kind of grainy. This is particularly bad with compensated data.

With compensated data, you have an additional problem. The problem is whenever you see a cluster, you always tend to draw data around it. So we tend to say, "This is different from these guys," but, actually, these are all the negatives.

It's hard to see them on a dot slide because you have got a substantial chunk of the

negatives clustered against your axes. The reason for that is that when you do this subtraction, we are taking a deterministic number and subtracting it from a random number.

Down in this neck of the woods is where all the noise lives. So every so often when you do the subtraction, you end up with a negative number because you're really looking down around zero. And it's a fluctuation on that zero.

So you can see this in uncompensated data. And it typically gets worse when you compensate data. So what happens is that things fail ungracefully. If you draw a contour around it, it looks like you have three clusters here when there is actually one only cluster.

One of the problems here is that the log of the negative numbers is undefined. The log of zero is minus infinity. So when you try to display this stuff on a log scale, there is no place to go.

The log scale is just a convenient dualization. The advantage of having high-resolution data is that you can do your statistics on the linear numbers. The log scale is very nicely displayed, but it has this failure in

that if you have got numbers that go down below zero, it chokes up.

So the question is, is there another scale that you can use that has the advantage of compressing the high-end data and expanding the low-end data but not blowing up when you hit zero.

There are a few. At the cytometry development workshop, which is an annual event for serious hardware jocks and software jocks, that is held in Asilomar, California every fall, this subject has been kicked around for a couple of years.

There were several approaches that were suggested. Jim Woods suggested that we do what they do in audio and use what is called a compander. The compander turns out to have the same problems as a log amp when you get to zero.

Mario Roederer, who is now at NIH, and Dave Parks and Wayne Moore and some other folks at the Herzenberg lab and Adam Treister and Tree Star, who was also in the Herzenberg lab, were kicking around a biexponential transform.

This is a transform that has got a lot of coefficients. And it basically uses hyperphonic science and you don't want to know. If you take

this transform and apply that -- so the reservation I had about this is that, you know, if you give people access to these coefficients, they are going to want to tweak them the way they tweak the knobs out of a machine. That's bad.

For sure, at least half the people who want to do the tweaking shouldn't be doing it. So if you can make a one size fits all or one size fits nearly all version of this thing, then this is good.

So this is actually done with a beta version of FlowJo. I think Dave Novo is working to put the same stuff into MCS Express. I don't know about Winlist. And logical is Wayne Moore's term for this transformation.

When you get here, you get the scale where you can accommodate a broad range of data. And your negatives now are from one cluster. The dotted lines show you where the negative values are, zero points. So everything looks good, looks like the kind of cluster you want to see. And we should all live happily ever after.

Now, whether it some weird things start happening on higher ends of the scale, I don't know. My approach, my counter-proposal to this is

if, for some reason, we can't get this out in the field, my counter-proposal is when you see a picket fence, just remember that that picket fence has a sign on it. And it says, "Beware of the data."

If this doesn't work out, then I think what we need is a data that grays out everything. You can put the numbers there, but you can't show the dots. That way nobody is tempted to gain on junk.

Bob?

DR. HOFFMAN: Joe Hart has been working with the guys at Stanford University.

DR. SHAPIRO: Okay.

DR. HOFFMAN: And they have been trying, basically tweaking the knots on that algorithm and see maybe what setting of that transformation works best and doesn't give you artificial sort of looking data on all sorts of different situations.

The next I think version of the software that comes out will have a compromised algorithm in it like this. So we won't have the picket fence or at least you will have the option of using a different axis to do this transform display.

DR. PURVIS: This is really, though, just an axis.

DR. HOFFMAN: It's just a display.

DR. PURVIS: So you're still collecting linear data, displaying the linear results on a transformed axis, correct?

DR. SHAPIRO: Yes. What this means, what this reflects is that the linear values at the low end, some of them are negative. And that is more so in compensated than uncompensated.

DR. HOFFMAN: But it's not just them. You're displaying data on a log scale. It can give you artificial peaks, --

DR. SHAPIRO: Yes, yes.

DR. HOFFMAN: -- even if there is no peak --

DR. SHAPIRO: Right.

DR. VOGT: It is true that that is still for the -- I mean, that still log scale in terms of the peak recognition would be the same.

DR. HOFFMAN: No, it's not a log scale.

DR. SHAPIRO: It's not a log scale. I mean, you notice that.

DR. VOGT: Well, that's not trying to go to 100. It is quite different than --

DR. SHAPIRO: It's a compressed scale, but the idea, the point is half the time until we had digital processing, the log scale reflected -- the log scale wasn't really a log scale because the log amp was not really linear along that entire scale. So the log data were always a little bit bent out of shape.

The point is that this is a display. This facilitates visualization. All data processing is done on a linear --

DR. HOFFMAN: What I am getting at is peaks wilt and look different than they would --

DR. SHAPIRO: The numbers are all the same. The data --

DR. HOFFMAN: No. The data is not what I am talking about. No. All I am saying is with beautifully normally distributed in log space and, you know --

DR. SHAPIRO: It will still log --

DR. HOFFMAN: They disappear. And it may be shouldered.

DR. SHAPIRO: As you know, it is really not --

DR. VOGT: Holding the low end.

DR. WOOD: It is kind of what you are

doing is you are moving in the low channels from something that is more linear looking to something that is exponential as you go further out.

Actually, in the histogram over here on the left, the buildup on the axes is really a function of the fact that the channels are varying in width. That is, any time you have a histogram where the channel width goes from something that is infinitesimally small up to larger and larger values.

Your peaks out in the middle, which looks like a separate population, is really the second part of the distribution. That is, half is on the axis. Half is out in the second population. And, practically speaking, that peak occurs about one SD away from the point where -- one SD away from the mean because your mean is actually there at zero on your noise.

And so that is really a -- the reason that you have the split there is because of the fact that the channels closest to the axis are so narrow. They're infinitesimally small. And then as you go away from the axis, as you usually go out, they become broader and broader so that they collect more --

DR. FISCHER: We understand that. Try telling that to somebody who is not used to it.

DR. VOGT: Well, I guess my question is I would be inclined to agree with Howard that it's better to not play these tricks and get -- I mean, if you look at that nice little cluster at the right end there, you really are not getting a good picture of the measurement process.

DR. SHAPIRO: No. You're getting a fine picture of the measurement process. The linear data are the same. This is a display scale. What we are saying is that these are the optical illusions. I mean, that is closer to the real state of affairs than either of the distributions on the left.

DR. VOGT: Because it is not bounded by zero?

DR. SHAPIRO: Well, because it shows the bad because it does not make a distinction that shouldn't be there.

DR. WOOD: If you think of the --

DR. MARTI: It establishes they're negative. I think this is a wonderful improvement, Howard.

DR. SHAPIRO: Well, I had nothing to do

with it.

DR. VOGT: But they're negative because the compensation algorithm is --

DR. SHAPIRO: They're negative.

DR. MARTI: Well, they're negative.

DR. SHAPIRO: The compensation --

DR. VOGT: They are negative.

DR. SHAPIRO: The compensation algorithm -- this happens with uncompensated data. You are more likely to end up with negative values with compensated data than with uncompensated data. But if you have values that are down near zero --

DR. WOOD: But negative values are real.

DR. SHAPIRO: Yes.

DR. WOOD: Once it restores, is this trying to take the baseline level of noise and put it at zero, which means there is stuff on the other side? You're trying to set your zero at the center of the --

DR. SHAPIRO: Bear in mind that if you are dealing with a scale of -- well, this goes from 100 to 10^5. If you know of an analysis that goes from a 4-decade window, a million modules down to 100 modules, this stuff is down in models. I mean,

there aren't that many instruments that are going to take care of 100 models.

DR. MUIRHEAD: Is it fair to say that this is a way of trying to visualize populations that are there without creating artificial ones? It's not really important for gating but that in terms of how you are going to quantify it, you're going to have an effect because you're not quantifying based on that transformer. You are quantifying based on the underlying data.

DR. FISCHER: Mostly for reporting out to people who aren't used to looking at the data and understanding where the problems lie.

DR. WOOD: It's a display function solely.

DR. VOGT: Right, but it will be used for data. I mean, that's I think --

DR. FISCHER: Yes, that is the --

DR. VOGT: That's how it could influence your results. It would in that case not be just a display tool. It's actually part of the analytical process.

DR. FISCHER: What we are dealing with here, I call it --

DR. WOOD: What this is is cluster

knowns for the human being. Okay? That is, the human being is expecting certain clusters to occur based on the antibodies that are being used. So if you orthogonalize everything, then you can think in antibody space. And a human can then do clustering and group things based on what they feel intuitively is going to happen.

Now, as far as a computer is concerned, it doesn't matter what the real orientation is. The computation on the raw data can be done in any space.

DR. VOGT: Given that the pathologist or cytometrist is used to looking at certain clustering displays and that's actually -- I have compared it to looking at tissue through a microscope. I mean, you see these patterns, and it is very important.

So is it true that this display would not too much disturb or perturb the people, that they will still be able to pick out their leukemic clusters in the --

DR. WOOD: Yes because what is happening here is if, say, for example, you were to have the luxury of having a full linear display and you could actually look at the big wraparound

display and pick out the clusters. You would see what you, too, want to see there.

What this does is it goes from at the lower channels to something that is linear because if you take the log function at the low channels, it's almost linear. And then it starts taking off and doing its bends, as do the compression.

And so at the low channels here, you are almost linear in your approach or your display. And then as you go further and further out, it starts compressing it. It actually goes according to the amount of information that is out there based on the standard deviation.

So at the low channel, where you need the fine resolution to see the small clusters, you have it; whereas, out at the far end, at the high end with high gate, the high values, you don't need the resolution out there. And so, therefore, you can start bidding things in bigger groups.

And so in this sense, it goes based upon close to the information content, if you will, of what is out there.

DR. LENKEI: What about the sources of error when it's from the clinical diagnosis? What will be the cost for us to know the process and

it's correct?

DR. VOGT: Well, that's sort of what I was asking. I mean, if you found that you have to pick out these clusters in order to --

DR. SCHWARTZ: It's going to look the same.

DR. SHAPIRO: Zoom display. At the bottom, you look in at 100 channels. At the top, you can see every data point is on the channels.

DR. VOGT: Right. I understand that. I'm just stating an operational question. Okay. Well, that's interesting.

DR. MARTI: Howard, thank you for that.

I wonder if before we take a coffee break, if we could get started on what was the next topic, which is QC. How are you doing now? How are you going to go about QC in your laboratories for clinical quantitative blood? Don't everybody speak at once.

DR. PURVIS: QC3 bead setup standard sessions.

DR. MARTI: Maybe this would be a good time for you --

DR. PURVIS: I would like to hear what everybody else has to say, instead of standing up

and saying what I do.

DR. SCHWARTZ: I think somebody should know what you do.

DR. MARTI: Everybody in favor of having Norm lead the discussion?

DR. SCHWARTZ: That's it. Yea, Norm.

DR. MUIRHEAD: Somebody has to start. Go for it.

DR. SCHWARTZ: Show us how it should be done.

DR. PURVIS: I would like to have heard some of your comments before --

DR. MUIRHEAD: You will. You will.

DR. PURVIS: I did all of this on the plane Wednesday night flying in here. So essentially what I did was took a bunch of my other presentations that I have given at other times, compiled them all, and they're up here now. So some of this is probably not going to fit right in with just QC. So we will scan through them real quick.

This is comments that I have had before. Why do we have to standardize? I think it is pretty self-explanatory. We want to be able to do it intra-laboratory as well as inter-laboratory

standardization.

Here is an example of why. CD8 FITC and CD4 PE measured on two different instruments. Can you tell me whether that is the same sample? Can you make any kind of assessment off of that? Quantitative? I mean, we could say something about percentages probably, but could we directly prepare the intensities off of this?

I think this is all about consensus here. Basically I base my decisions and my interpretations on the historic data that I have seen, what my personal experience is. Yours is going to be different. So it's a group of people sitting around a slide looking at the scope and, yes, all in favor.

Next one. Well, here is the data we distributed from those same two sets of plots in a quantitative space. It's putting it on a quantitative scale: a, b, c's.

What we have there is the same sample run on two different instruments with two drastically different instrument setups. So it kind of goes back to what we were talking about with Jerry's stuff the other day. His ended up that we had different samples. And I didn't catch

that in his introduction to the data. This one truly was an instrument setup issue.

Just by running MESF beads to knowing what that piece of these antibodies were, I can do this. Okay? And that's what we want to be able to get to so we can compare data.

DR. VOGT: So, Norm, then the blue data was set up with presumably a lower PMT so that the resolution --

DR. PURVIS: What we lost was the negative information. What we lost was the negative. Therapeutic capabilities, this is why we are looking at quantitation. And we have therapeutic monoclonals, cell signal, drug pathways.

I think we just need to be focused on surface markers, but there is also a distinct need to be able to do quantitation for cytoplasma as well and nuclear. So there are a number of important issues. You have got to standardize.

I would go so far as to say we need to standardize how we do our instrument setup, but if we are going to go to absolute quantitation, as I showed you a while ago, if what we are interested in is the positive expression and not so much

negative is negative, then, even by applying quantitation, we can standardize. Okay?

I am going to go back and say this over and over again. There has to be agreement in the standards. I know that we can dead with bias. BD has a bias in one direction for PE and Bangs has a bias in the other direction for the PE, but I think that there needs to be the true absolute quantitative value that we are working with so that apples are apples.

This here, well, I don't know. Yes, it would be great if we could define what the lower limit of quantitation needs to be and then set up our instruments and standards capable of resolving that. That is into the future. Okay?

DR. VOGT: But, Norm, suppose I said, actually, the lower one were closer to the last one and getting closer to the middle one and furthest away from the top one.

DR. MARTI: Yes. Why is that? Everyone wants to set up their -- well, you know, from a regulatory standpoint, it is the old issue. The minute you mention the word "standardize," then you take flexibility out of the system. Maybe that is what people don't even know that they are

opposed to.

DR. VOGT: I think that is a major part of it. I think those are three very good pullout points from this conference.

DR. PURVIS: So, as I've given some other talks, essentially what we have got to do is concentrate in this one. Set up standardization, quality control and quality assurance programs, quantitative reagent quality control and standardization. The standardization there is something. It's not absolute standardization. It is how we are going to evaluate it; and then procedural standardization. We have discussed that numerous times already.

DR. VOGT: As in preps and that kind of thing?

DR. PURVIS: I put this slide up mainly to get across the point that we are a laboratory that has seriously been performing standardization for a long time. We have multiple sites. We have multiple instrument platforms that we are working with.

We are in a process. Currently we have got 15 Epics going, but we have 6 FC500 installed. And we're in the process of replacing the XLs with

the FC500s for a number of reasons that have already been pointed out here with the digital processing, the 20-bit linear data access. There's a number of reasons that we wanted to be able to go onto this platform, not to mention resolution, sensitivity issues that we have got.

Anyway, the other thing that we do and that I have been required to do is to go out to principal investigator sites, go in and set up their instruments, and allow them to participate in some of the clinical trial work that we do.

So I have got to set up their instrument and also train them on the procedures that we use in the actual processing of the samples because it is not just instrument standardization. You have to standardize the procedures. I think that is what we are going to have to do here.

But not all assays are standardized the same way. So we are going to have to look at a number of variables. So it does become experimental. It is an experimental process, each one.

DR. VOGT: Just to stop there for a second, where do people learn how to prep? I mean, basically in the laboratory they happen to start

working in, I would guess.

We haven't talked at all in this conference about there are a lot of didactic exercises in instrumentation and setup and none in preparation.

DR. QUINTANA: What happens, Bob, for some of the training -- BD does the same thing -- is that they have customers like that would be running the automated CD4 systems. And they come for training. They get trained on those processes, what they do. They might get training in general but then on the individual.

So I would imagine when clinical assays, clinical quantitative assays, come out, that we will be training people, purchasing or using, starting to get new instruments and stuff like that, that they are going to be trained at the manufacturers also on the specific methods that they use for setup.

DR. VOGT: I think that would be very important for deliberations of this type of thing to have communications or some impact on those training programs. And then also the people who already have prep training and buy a new instrument probably don't either get that much or get trained

that much when they come to learn about their new instruments.

DR. PURVIS: And I think one of the other things that needs to be pointed out -- and I normally do this -- I can't use your automated instrument setup. Because I have BD, I have Coulter, they don't give me the same instrument setup. So I have to go to another program.

DR. HOFFMAN: You use the BD kit to set up the instrument and try to use the same kit to set up Coulter.

DR. PURVIS: And you're automated, your compensation, everything that you all are doing, I've got one over here and one over here. How am I going to make these instruments look the same?

DR. FISCHER: Is that because we don't have one uniform standard that everybody should be using for setup of instruments?

DR. QUINTANA: They are different protocols.

DR. PURVIS: And there's a number of differences within instruments.

DR. QUINTANA: They're optimized reagent systems and software, other systems.

DR. PURVIS: I am not going to take

credit for it. The man sitting right back there is the one.

DR. HOFFMAN: I didn't take credit. The fact that you set up systems for each of their instruments, you need to set up a system that is probably --

DR. PURVIS: It covers everything that I can possibly go into. So there are still some other instruments that are sitting out there that I have to go into principal investigator sites and set them up as well. So we have had a tremendous challenge in doing this.

We will go on to the next slide.

DR. MARTI: I don't think I would hold my breath waiting for the manufacturers in their training courses to do much about standardization procedure. I would think about going to the lab where the procedure is being done and seeing if I could transport it from that lab back to mine.

I think given the experience that we have had with Abe's course, both in this country and internationally, that the kind of stuff that you are talking about, when I am in the setting for those courses, the questions I get from the users would certainly suggest that those questions

weren't answered when they went to the manufacturer's course, such as "What is the difference between a log scale and a relative log scale, relative fluorescence scale? Which scale are you using?"

DR. PURVIS: Which scale am I using?

DR. MARTI: No, no. When you just ask, most people don't know. I mean, 60 percent of the AIDS cohort -- that was a factor that emerged -- did not know which scale they were using. They still don't know. They use whatever the default one is.

DR. PURVIS: There is so much terminology that is used there, RCI --

DR. MARTI: Oh, yes. They keep inventing new ones.

DR. PURVIS: I think we have got to come up with a name so that we know whether we are talking about channel numbers, log channel numbers, or actual fluorescence intensity, I think, simplifying those terms down so that we are at least talking about either intensity or channel number.

When you're in the linear scale, they're one and the same. It's a relative

intensity. When you're in a log, log channel numbers do not reflect the actual relative intensity, fluorescence intensity. We have got to come up with some consistent terminology to use.

DR. HOFFMAN: I think we should stop using the term "log channel number." It's really outdated. We don't need it anymore. We know that in the current software, if you are going to be using log amplifiers, the data is as linear units.

DR. PURVIS: We still use log channel numbers in doing our QC.

DR. HOFFMAN: We do, too.

DR. VOGT: Well, Bob, I think this will be an unnecessary conversation in two years or so, but back when everything was analogued, I objected to the use of linearization because it made this presumption in channeling.

I thought the raw data is how many events fell in this particular bin after it had been log-amplified and that was the real data. And it's all coming out on this convenient linearization is misleading.

Now we have gone through a transition point here where some analog, some digital. When we come out the other end in full digital, then I

think you would be exactly right. And I guess we're not far away from that now. And that may make things a lot easier.

From the standpoint of fluorescence intensity, the definition of fluorescence intensity that is in the NCCLS guideline is the reading on the instrument. I mean, that is actually what you get. It can be a needle going from zero to whatever.

DR. PURVIS: But that's some relative intensity.

DR. VOGT: They're all intensities.

DR. PURVIS: That needs to be pointed out, though.

DR. VOGT: Well, the fluorescence intensity is --

DR. PURVIS: If I could change that number that you've given in the result by changing the voltage --

DR. VOGT: Right, but the fluorescence intensity still is the instrument reading. The fluorescence radiance is the actual amount of fluorescence. And how the two of them relate is the subject of quantification. That is what you are trying to establish.

Any reading off any instrument is a fluorescence intensity. And you don't use the term "relative," but they are all relative. The log scale is relative, too. It's just that it's probably quicker.

So all fluorescence readings are relative. And they're all fluorescence intensity. And then the way that relates to the actual amount of fluorescence is --

DR. PURVIS: What did you call it?

DR. VOGT: The radiance.

DR. SHAPIRO: Actually, most of the scales of optical measurements are really photoelectron scales because they're what comes out of the detectors.

DR. VOGT: Right, but that is still considered fluorescence intensity.

DR. SHAPIRO: Yes, yes.

DR. VOGT: The equations that put those things into --

DR. SHAPIRO: That's true, but we are not getting into figuring out how many watts or even how many photons there were because there is a quantum efficiency thing there that we --

DR. VOGT: Right, but there --

DR. SHAPIRO: Well, yes. What we know is coming out, we know that somewhere in the detector, there are photoelectrons coming out. And everything proceeds from that.

DR. VOGT: Right, right.

DR. SHAPIRO: I do think it would be -- I mean, when I took a Coulter course years ago, there was no discussion whatsoever of the scale per se, what is it that you are measuring. And I know that none of the manufacturers have time with new users particularly to train them in optical physics and electronics and so on and so forth. There was no discussion of what this scale means.

DR. HOUTZ: I think with the methods in the software dissertation, you see the educational benefits. I mean, it's a whole week's worth, and it's pretty intense. But it's still much more basic than what we are talking about here.

DR. VOGT: Well, is it all basic or is it just more useful? See, I have always argued --

DR. HOUTZ: Specific to the product, too, though.

DR. VOGT: And it's completely to the product. I think this stuff can be presented in an hour, the basics of what it is. I don't think

people even know when they come to this course what the -- I don't think they think of it as a fluorimeter. They think of it as a flow cytometer.

I think one hour would be plenty of time to explain the flow cytometer as a fluorimeter. That would include and probably end with a discussion of the scale.

DR. HOUTZ: To a basic user?

DR. VOGT: Yes, yes. I think people are unnecessarily afraid of this. You know, it ain't the question of the origin of the universe. It's just a tool.

DR. HOFFMAN: I was really talking about the physics, about what is laser, what is NP. There are some slides. We can introduce those.

DR. VOGT: Right.

DR. FISCHER: The thing is that most of the education programs, -- and I have been through several of BD's, one of Cytomation -- the main focus is on how can you get back to your lab and plug a sample on that instrument and get data out. Whether you understand how it is doing it or not is not the big thing.

I would argue from a lot of the users I have worked with I don't want them to understand a

lot of this stuff because they are going to want to start monkeying around with instruments they have no business even touching.

DR. VOGT: That becomes a self-fulfilling prophecy. And you wind up with a dumbed-down group of people operating your instruments. I argued against that 15 years ago, and I will argue against it now.

DR. STETLER-STEVENS: We talked about details of education --

DR. VOGT: Wait. Mary Alice, I think it is a forum to talk about it because this is what you are trying to do. And it can start with the pathologists would be my suggestion, that they don't know what they are doing either.

DR. STETLER-STEVENS: I think that the place to discuss it -- we do need more education in so many areas, period. But are we going to solve this problem right now?

DR. VOGT: No, but here's the thing, that it's all quantitative flow.

DR. STETLER-STEVENS: Then let it be more in education.

DR. VOGT: Everyone is saying, "Oh, God." It's all quantitative. I mean, we're

putting these clusters in places because it's quantitative. If it wasn't quantitative, we wouldn't have clusters.

And I'm saying that you can begin there and in an hour, you can get it over with. And if you did that, you would have a community that understood that the cluster doesn't appear there because it's a cluster. It appears there because this is brighter than this and it was measured by the instrument that way.

It doesn't matter if it is PMT or an APD. I would spend no time discussing PMTs.

DR. FISCHER: Yes. We're not arguing with that.

DR. VOGT: Right. But I never heard it presented that way. And, actually, I still don't, you know. And in the NCCLS process, we kind of pick through it, that, you know, this isn't that hard. It should fall out. As Jerry always said, the instrument should fall out of the equation.

DR. SHAPIRO: Well, at this meeting that you very nicely got me sent to in Belgium last week, this is a meeting to try and define standards for biomedical metrology of all kinds, flow cytometry being a relatively minor thing here.

What is metrology? Metrology is measurement science. The answer is that everybody who is doing research is measuring something. They all think of what they're measuring, but they don't think of the common measurement issues. So nobody knows what metrology is, but everybody is measuring something.

DR. FISCHER: All the metrology we did when we worked for a prior company, everything got traced back. Whether we measured temperature, whether we measured weight, whether we measured volume, everything went back to the standard that is kept at the National Institute of Standards and Technology.

Somewhere back along the line, someone made the decision that this is what we are going to measure everything by. Time is involved in that as well. If you look certainly in any GOP laboratory, it ought to have the little stickers on things that say that they can back to a NIST-traceable standard.

There is nothing for setting up our instrument that goes back to a NIST. This is instrument qualification and operational qualification, instrument and operational

qualification for these instruments. We don't have that for a flow cytometer, although I understand from BD that they're now going to be putting one of those out.

We don't have that. And that is why a lot of the differences that we have come up with now are theirs because we don't have any standards that have already been set up with the instruments.

DR. STETLER-STEVENS: Can I say that for education purposes, why don't we, you and whoever else wants to, come up with a list of what you think needs to be addressed in education, maybe not the first week? When you're just starting to learn how to turn the flow cytometer on might not be the best time, but maybe at the cytometry meetings, we should suggest to manufacturers courses that we think would be beneficial or we could even within the format of the society, the Clinical Cytometry Society. They have educational workshops. We could address this issue by forwarding it to these groups what we think needs to be addressed in education.

DR. FISCHER: There's a lot of enough local user groups out there that they could run a half-day educational meeting. Instead of always

having Howard come down and present data to us, they would have Howard come down and teach us some of this stuff.

DR. SHAPIRO: The idea is that the book has always been designed to include pretty damn near everything you would want to know.

DR. FISCHER: Yes, but, you know, Howard, an awful lot of people won't pick up a book.

DR. SHAPIRO: Well, I suppose education --

DR. FISCHER: I hate to say it. I love to read, but I hate to say it. I pick your book up mostly when I'm sitting in the lab going, "I don't remember that." Then you go find Howard's book. I'm not taking it home with me to read.

And a lot of these people, that's the only time. They're going to pick up the journal article that is concerned with their science before they are going to pick up the book to figure out how to make their science work right.

DR. SHAPIRO: There were a few people who wanted it to be an electronic book, but most people didn't. So in the introduction there, I said, "The bottom line is you can still read it in

the bathroom."

DR. STETLER-STEVENS: So, Randy, you are on the Education Subcommittee. Let's move on.

DR. VOGT: See how easy this is, Norm, for you to stand there.

DR. SHAPIRO: Who else needs to check out?

DR. STETLER-STEVENS: Should we put Howard on it, too?

DR. VOGT: All right.

DR. SHAPIRO: Well, balance me with Alice.

DR. MARTI: Let's take a break.

(Whereupon, the foregoing matter went off the record at 10:08 a.m. and went back on the record at 10:40 a.m.)

DR. VOGT: This is it, Norm, is that right?

DR. PURVIS: Yes, sir.

DR. VOGT: And so where did we want to go on this? I've forgotten. Yes. We have gotten through what, four slides? Is that right? Eight? Hey, we're really moving here.

DR. PURVIS: I guess continuing now, I agree that education is something we need to work

with. There are a number of different setup options available to us. BD has one. Immunotech has one. I'm sure Tri-Dimension has one. Ortho had their own. So everybody has their own idea about how you are going to set up.

One of the problems is that they are going to teach you their method, but that doesn't work when you have multiple instruments, multiple assays that you are working on.

Dr. Schwartz came up with QC Windows -- how long ago was that? -- as a way of standardizing our flow cytometers. I think it is a very good approach, an approach that we have been using forever now. We have modified it some, but for the most part, it is the same general idea that Abe was putting forth two years ago.

So we do an initial instrument setup using QC Windows now from Bangs as well as other labeled beads and stained normal donor leukocytes, healthy volunteers. I won't claim that I'm normal or anybody else in my lab is normal.

We go through an initial setup. We lock everything in. We don't set up our instrument unless it's -- we develop a very good QC program around our instruments to verify that our

instruments are within our specifications, performing properly. And that is all based on our daily QC.

We use here a combination of QC3 beads, full spectrum beads, CalBRITE APC beads depending on whether we are doing a Calibur where we use APC signals; FC500s, where we are using the flow set beads, Flow Set 660s and 770s. So it is customized, but everything is truly being based on the standardized setup in many of the different channels as I possibly can get a standardization problem.

We do multi-color staining and do our compensation on that, verify our compensation on a daily basis. So not only are we looking at our beads and knowing that our compensation is turned on when we are looking at the beads and they should be falling in a certain range, but we also go back and multi-color the same sample so we can verify that that is good.

Then we also took --

DR. STETLER-STEVENS: Do you approach this --

DR. PURVIS: A combination of both. A combination of both. For the most part, we found

in our clinical area the samples that are -- because those are in PECy5, Cy7, our procedure was to -- the clinical trials area is essentially freshly stained samples that they're doing the basis off of because the samples are generally --

DR. LENKEI: Stained for five days. We stain always on a Monday, but it's for the four-color. It's four colors. I would check repeatedly. It's no change for this type.

DR. PURVIS: And I've seen that with this. I would say you could do that without any problems. To tandems, no. Even after they have been put into a fixative and run, for the most part, 24 hours later, you can still run them in the compensation that you see initially compared to the 24 hours. It doesn't change.

But you will start seeing that tandem degradation occur once you go beyond. So there we do make sure that we are dealing with fresh stained samples or something that is no more than 24 hours old.

DR. TAMUL: Norman, do you use normal leuko cells or do you do any of the commercial controls?

DR. PURVIS: The clinical lab does run

some commercial controls.

DR. TAMUL: Like CD checks?

DR. PURVIS: Yes, CD checks or the -- what are they called?

DR. TAMUL: Immunotrope.

DR. PURVIS: Immunotrope. Primarily, it really goes with our lymphocyte cell-setting stuff that we're doing for the leukemia/lymphoma. There is really not something that is available for us in that.

There are some stem cell controls that we do use as well that are standardized. They have a percentage that we ought to be hitting. We are using those, but that kind of goes along with the assay QC and not really the instrument QC.

DR. TAMUL: Okay.

DR. PURVIS: Okay. The other part of what we do is our linear characterization. We can do that either with quantitative or qualitative beads. So I've listed those up here. And I've put Quantum 1000, but you can also use the QuantiBRITE beads, trying not to just show what I do but give you all your different options.

That's essentially our setup in a nutshell. So here is our modified QC Window setup.

So here is our modified QC window setup, pros and cons. What we are trying to get here is a common window of analysis on all of our instruments and as many of the different detectors as we possibly can.

We are going to base our compensation on biologic samples. I have a problem with using beads to base my compensation. I think for the most part they work all the time or they work most of the time, but there are cases where I have seen the bead compensation methods not work. So I tend to stay away from that.

We do one instrument setup from all biologic samples. And DNAs fall outside of this because you have to run DNAs special. Some of the cell line works that we are working with, you can't run cell lines at this high setting. Your cells' auto-fluorescence will be up on the second decade, and everything else is off-scale.

So in some cases, we do have stuff that we will run at some other settings, but for the most part, we establish our settings. We do our QC at those settings, verify our compensation is working at those settings, and that is what everything is being on that.

DR. LAMB: Where are your -- when you

say, "follow-up samples," that thing that comes to mind is that --

DR. PURVIS: That's what we use.

DR. LAMB: You have it done every day?

DR. PURVIS: Yes. And then we also use some of the patient samples that we do. We process some 20,000 LL beads a year just in the clinical lab. And then we have other patient samples coming in through all of the other labs. So we have a steady source of samples that we go to. And we also go back and reconfirm by re-staining them the next day if we needed to.

It begins to address this approach. The modified approach, using the beads, the linearity beads, begins to address our resolutions. I think that Jim and Bob have both worked out a spreadsheet for actually looking at this in a much better way using the rainbow beads or some other beads to be able to characterize it.

So that is something that I would like to incorporate in my QCs to look at that and see if maybe not on a daily basis but at least on a frequent basis, go to it and see if my instrument is changing because your low-end resolution is very important as well. So we want to be able to go

towards that, but we haven't at this time.

It allows us to do four-color instrument setup. Actually, that is moving to five-color now that we have moved to the FC500s. We're doing seven-color on our Altra. So this does work. We just have to have all of the materials.

Colors. It does not standardize your background. Okay? You can have an instrument -- the Ortho instrument was one of them. The XL is one of them. The low-end resolution, sometimes we're pulling the negative cells out because we have really standardized on the high fluorescence intensity and tried to hit a mark there. And it will pull it out. That is something that you have to be aware of and look at. If your instrument is bad, you need to address it. And it's time-consuming.

The good thing is we do it once. And I have instruments that have stayed in this QC setup for over three years without having to go back and make other changes, even after having a major PM.

As long as the alignment has been put back in, they come right back into place. As long as you are keeping your instrument good and clean, you don't have flow problems, you don't have

fluidics issues that are affecting it, this does work. So I am not running an automated system.

Yes?

DR. ORFAO: Do you also mean that they remain stable for three years for a standard --

DR. PURVIS: Our standard is standardized as well. We standardize our scatter off of the lymphocytes and --

DR. LENKEI: Even when you have an instrument service?

DR. PURVIS: Yes. If the engineers do their jobs properly, yes.

DR. LENKEI: Yes, they do that.

DR. HOFFMAN: Do you leave it the same and then you monitor what the --

DR. PURVIS: No, I do not adjust. So I am expecting it to fall into an acceptable range. And as long as it is doing that and my compensation is also working properly based on our verification, then I am confident that the instrument is good to go.

You will have some slight variations, but for the most part, from those slight variations and drops, temperature can have effects. There are a number of things that can have an effect here.

The absolute change that it causes into the compensation is not enough to warrant going through and resetting it up for our clinical applications without a doubt.

Some of our more quantitative assays, there have been some questions as to whether we should be adjusting that.

DR. DAVIS: How do you define your acceptable range?

DR. PURVIS: This was something that we defined years ago. I being an engineer would like to tighten up our acceptable range more than everybody else, but it was based on looking at the variance in the instruments, a very good instrument, and then defining it. I think we are using plus or minus 15 channels right now, but that is log channels.

I think that at bright intensities, those log channels are problematic. So I would rather go to the intensities and say, "We are going to allow this much of a variance." I would like to tighten it up. I would vote for five or ten.

DR. HOFFMAN: Well, that is going to depend on whether the manufacturer can actually make the instrument and has designed it to be that

stable over whatever you are offering.

DR. PURVIS: That has been a problem, but I would still think that tightening up probably for me would be something that I would suggest I would like to see immediately.

DR. DAVIS: So you can look at it statistically in terms of establishing 2SD replicates.

DR. PURVIS: We have data that we can go back and do that and actually tighten it up based on our historic data. And if I were to do that based on my historic data and call it a 2SD cutoff, then we would probably be down around a five range.

The clinicians really felt like I was tightening it up and taking an instrument off line, that from their perspective, they weren't seeing enough variance in the data or they couldn't even see the variance in the data to warrant it.

So on the quantitation side, maybe there is a need to tighten it up and expect it to be more stringent than that, but we are probably talking about 2SDs at the five-channel range. It depends on which channels you are looking at as well.

If you're multi-laser and time-delayed, that has been problematic. I definitely couldn't expect that on my sorters, I don't think, multiple laser sorting.

DR. HOFFMAN: Just one other question.

DR. PURVIS: Sure.

DR. HOFFMAN: Do you also leave the compensation setting the same or do you change that each day?

DR. PURVIS: Don't be silly. It's all kept. It's locked in.

DR. HOFFMAN: So then you just check that you have got appropriate compensation after?

DR. PURVIS: Right. Now --

DR. SCHWARTZ: In fact, if it meets his criteria, most likely the compensations are okay because if the compensations weren't, it would screw it severely. And then he checks it again with cells to make absolutely sure.

DR. PURVIS: And we're going to go through these quickly. If you want me to comment more, than I will. But the initial instrument setup is truly based on a fluorescence PMT setting using QC Windows, where I have a target channel for a bead that has been given to me in a COA. So this

is where I will still be setting this instrument up to.

One of the things that we do in our lab is when we get in a set of beads, we compare it to the old set of beads that we have been running with to make sure that we are not changing our instrument setup based on the manufacturer has made a mistake or something has occurred during transportation, which has happened in the past. So we verify compared to the old lot as well as our historical values.

We know that we should be running very consistent. So we adjust the voltage to obtain a target channel. And this is initially done with the compensation turned off. So this is the initial instrument setup.

We have done the fluorescence intensities now. We need to get the scatter detector settings standardized as well so we base that on our lymphocytes. Here we are using healthy volunteer whole blood. So for the most part, we are sitting in a very consistent pattern here. Most everybody's lymphocytes are good enough for us to work with.

We have our PMT voltage gain settings.

We have our scatter. Now we need to go through and look at compensation. Now, this is where I will differ from Mario Roederer and some of the other people that are out there because I have pathologists that I have to make happy.

When we do compensation, for the most part, they don't want to see the flare at the end resulting in sales in a second decade or third decade or whatever it ends up being at. So they won't need to bring it down so that it looks orthogonal and everything has a flat top to it.

DR. HOFFMAN: So you're overcompensating

DR. PURVIS: I am overcompensating to a certain extent, yes. I walk a fine line with them. I try to still have some events moving up into the second decade. And then realizing that for the most part, my actual test antibodies are going to be much dimmer than what I set up here, I don't have to worry about that, seeing that flare.

But I want to make sure that if I did get something biologically, plasma cell or whatever, that really jumps out there, that I am not going to make the pathologists panic because they have seen something that is like a 56-positive

or whatever we have got, the combinations, going in there. They chase their tail, and then they come after me.

All right. We do a biologic compensation. I have listed here how we approach that is to get a biologic continuum. Each tube has CD3, CD4, CD8 in it, ECD, PECy7, antibody combinations that are Ortho combinations that we might be using.

What you end up with, b-cells, nK cells, and RT-cells, it makes it a very nice biologic continuum there. So as we start to adjust down here, I can see what my compensation is actually doing. Okay?

Some kinds of ideas of what we were talking about before. You need two peaks or two populations to base it on. I've got essentially a continuum that I can base it on.

Go down again. The previous one I was under-compensating. This one here I have overcompensated. I know all of you are saying, "Why did he make it so bright that they're going out in the last channel and piling up out there? That is causing all kinds of problems."

I want to see them out there. I want

to know what is going on out in that last channel or the channel before I hit the last channel so 1,022, I want to know what is going on out there.

I have to ignore those that have piled up in the last chapter. I don't know what their intensity is. The instrument doesn't know what the intensity is. It can't properly compensate. So I ignore those from the standpoint of looking at mediums or whatever and trying to get my compensation set up perfectly.

Next one. This would be fairly close to what I would call our compensation. I have gotten my team pass to accept that if they get something out there at this end, there is going to be a tail up there. They are really looking for a distinct population above that now. So they are accepting it.

You can see here what it is in the last channel. They are tailing way up there. They have to be aware of it also because that population, if you did something like a plasma cell that goes off-scale, it will look over in another.

If you are just looking at single-parameter histograms, it will look like you have got a population sitting out there. And you

have to go back and look and see if it is truly a compensation issue where something has gone off-scale and you haven't properly compensated it. That's essentially how we go through the compensation.

Next slide. I put this one in here because I think there is a lot of confusion on the users as to, man, let's use this log bias. Let's put this artificial signal in here and use that while we are setting up a compensation because it makes it look so much better.

DR. HOFFMAN: Is that an offset that you put in your software?

DR. PURVIS: Well, you could actually do it on the Coulter software. You can turn on a log bias. It's not stayed down into the list mode file.

DR. HOFFMAN: Is that an offset on the --

DR. PURVIS: Yes. You can also do it in Winlist. And it's just essentially they're taking and redisplaying it into the events that are down on the axis. They're redisplaying up into this first decade. So this is our normal display. This is with the log bias turned on.

If I start compensating down, you can see that here, all of a sudden, I started getting events that had gone on to the axis but are being redisplayed back out. So it's showing a double population there that really doesn't make any sense.

Here that looks beautiful. I mean, I would love to see compensation that looked that way. If you look at it, really, what has happened is I have got a huge number of events down onto the axis so we don't see them there any more. And they have been redisplayed here.

Same here. I still probably significantly overcompensated there based on what I am seeing. This is probably getting pretty close. So it is somewhere in between this one and this one is what I would really be -- these are slides I made a long time ago, before my pathologists kind of loosened up a little bit.

Next slide. Data QC. At this point, we have got everything set. I can go through this setup in about a two-hour process. Now I would average what my daily targets need to be for my daily QC program.

So now I am going to start running with

my voltages set on, turned on, compensation turned on, everything set up the way it needs to be, or how I am going to be actually running the instrument.

I am going to start running my bead sets back through. I am going to do this over multiple days. And I may even make multiple measurements each day. When I establish what my target range needs to be, what my mean of that needs to be, and then we allow that plus or minus range to deal with, it is essentially just running those through and then also running our biologic samples and verifying that we have a consistent compensation that is going to work, there are not going to be any problems. And then this establishes our daily QC specifications that we have to be on a particular instrument.

One of the things that I think a lot of people have made a mistake of doing -- and I know that this is the case in some of the principal investigator labs that we have gone into -- is they will see this. They have a target range that they want to hit.

All of a sudden, something happens one day. They come in. They turn on their instrument,

let it warm up, and it doesn't hit the numbers that it is supposed to hit. Automatically because we told them, "You are going to follow this if this is outside of this, you have got to reset up your instrument." They are going to go through some simple troubleshooting issues to see if that is what is going on.

Most times or one of the big problems that we were having was bleach was left in the line or something else. And the beads would hit that bleach before they made it into the flow cell, and things were all messed up.

So there is some simple troubleshooting that you can do. In 99 percent of the time, just by going back through and flushing the system, doing a prime, clearing any bubbles that might have gotten in the flow cell or maybe it was our sheath tank cap wasn't screwed down tight enough and we weren't pressurizing to the same extent.

Simple troubleshooting, 99 percent of the time, will get us right back in our range, and we can keep going. So that is why I am saying if we clean our instruments and stuff, there is no problem getting this to run for a long term.

Next slide. We will go over it. We

don't need to go over this. You all know the differences.

Next slide. Okay. Resolution and sensitivity. This is just a comparison. One of the things, when you are setting up an instrument, you have got to know what you are dealing with and what you are trying to be able to measure.

So here are some rainbow beads run on the primary instruments that are out there. I know that LSRN and LSRII are out there, but I don't have access to those. So I ran the ones that I had access to minus the FACScan.

You can see FACSCalibur results, the populations. XL has a real big problem down here in the low end resolving the populations. FC500 is actually doing a better job than the Calibur for this particular caliber, but this is very nice resolution. I am very happy.

So when we are talking about quantitative, I want to be working on this instrument or this instrument. Low-level I don't want to be trying to do it on the XL here.

DR. HOFFMAN: Have you cross-Calibured the rainbow beads to -- I mean, those differences in filters can give you -- because of the rainbow

beads

spectrum, especially in

DR. PURVIS: We see the same with Quantum beads, the FITC. I don't think he calls them Quantum anymore. I think they're going by the catalog number or something.

That can have a problem. That can introduce some --

DR. HOFFMAN: I wouldn't necessarily call it a problem, but just for making comparisons, especially across instruments that are --

DR. PURVIS: I know that I would have the same filters in these two. Correct me if I am wrong. Jorge is here.

DR. HOFFMAN: Is it 30/30?

DR. QUINTANA: It's 525, I think, times 10^{15}, yes.

DR. PURVIS: I'm not sure what the caliber is. It's been such a long time.

DR. HOFFMAN: 30/30.

DR. PURVIS: So you're actually picking up a little bit broader ranger here.

DR. SCHWARTZ: That's when you get a better resolution on some of them.

DR. PURVIS: All right. But, anyway,

there are other routines that we can actually look at and can actually calculate. That will give us the A and B value that the two of you all have worked out. I would love to be able to start implementing that as part of my initial instrument setup and QC programs.

Next slide. Log linearity. I think it's important to know what you are working with and how well you can use it for quantitation. Here I have taken the rainbow beads and just used the old technique that I want to start with a low voltage and I want to increment my voltage up over and over and over again.

It should have eight peaks here. I've gated it, each of those peaks, off of one of the other detectors. Now I am looking at it over here in FL2. So I don't adjust this, and I am only adjusting FL2 up. So I can identify each of these eight populations based on the one detectors and then follow it on the other detector as I walk it up.

So I get very, very good information as to the median values on those as I walk it up. And at some point, I am going to start going off-scale. I will be walking some of the populations off.

Next slide. Doing this on these three instruments, this is the type of information that you will see. This is one of the problems that we have with the old log amplifiers. It approximates a linear process, but it is not linear.

What I am displaying here is the delta between those peaks, Peak 1 and Peak 2. In fact, this one was Peak 8 minus Peak 7, 7 minus 6, or 6 minus 5, 5 minus 4. I did it on each one of them. So I have got multiple plots here, and I can see what they are doing as they walk through.

The XL has a problem. Down here when they were in that first decade and a half where I had problems with resolution, you can see that I have got some weird behaviors going on. Sometimes I would come across XLs that bode down. Other times I would find ones that bode up. So it just depends on what is going on in that calibration that they have on the detectors.

Here on the FC500, you are getting pretty good linearity across the entire range.

DR. SCHWARTZ: When you did that walking, was it like one volt? At every volt you made an increment? It almost looks like that.

DR. PURVIS: No, no. This was five

volts.

DR. SCHWARTZ: Five volts?

DR. PURVIS: Yes, worked it up. I think it was 5 volts, may have been 25. There was a lot more on this one, but I did very small increments so I could actually see what I was getting there.

This here causes a problem with our quantitation because our four bead types that we are using to get our calibration line, where you are on that log scale can affect, give you a little bit of a wobble in, your calibration curve.

So you may get one set of beads that will give you this but have a different set of beads that when you have different values, they fall a little differently on the scale, it causes problems.

You have the same problem going on on the XL if you have a peak that is falling in that lower second decade. It's getting in there. It causes some problems.

The FC500 so far I haven't been able to really see that. I do have this as being shown slightly. And I think that is more a function of where I was in that first decade and whether I was

really resolving that population, I was getting a mass measure of the median channels, yes.

Next slide.

DR. VOGT: Norm, before you leave that one, that's an old technique and looks really good. I just am curious about whether the folks here, particularly engineer types, would agree that this still may be the state of the art or way too preliminary.

We did guideposts. How do you do linearity? Of course, we use neutral density filters because you can get them, and they literally have eight or ten decimal places with beautiful density value. And they are NIST-certified. So there is reference material for that.

And you can put those new Kolinsky filters in front of you, Dector door in front of your expectation energy. But then the discussions with Bob pointed out that you are also filtering out the noise, the optical noise, that you would get from the particle in the flow cytometer. I guess especially toward the lower end of the curve, that will show you a linear response, but it won't have an anomaly there caused by a noise

contribution. Is that correct?

DR. HOFFMAN: Also, to do the whole scale, you need an awfully expensive instrument, and I don't know if you necessarily want people going in and trying to --

DR. SHAPIRO: It filters a degree or two. Generally speaking, when the stuff is with the optical elements in the back --

DR. VOGT: Then you screen things out.

DR. SHAPIRO: Even though you may have many decimal places --

DR. VOGT: Okay. So that's --

DR. SHAPIRO: This is a nice method. When you start getting down to the low end of things, you may actually get back --

DR. VOGT: That was going to be my next question. Are there some PMTs that you can have responses that your PMT is constant across your different voltages you are studying?

DR. SHAPIRO: We are looking at it. Essentially, the channel's difference on the log scale is a ratio between fluorescence intensities of the two peaks on a linear scale.

DR. VOGT: Right.

DR. SHAPIRO: That is going to be

constant. So that is what should be constant across the board. The ideal response curve is one of the most horizontal lines.

The thing is that there are different kinds of log amps. The kind of log amps that are in Calibur are basically the modules. One takes over from another. That's why you have the waves across the top.

On the FC500, where the whole process is digital, then that's pretty close to the horizontal line.

DR. VOGT: So we, then, agree that if we are evaluating linearity response on a flow cytometer, this is probably still the best available method?

DR. SHAPIRO: The way I see it. And the thing is that some log amp response curves are stable and some are not. So if you have a stable log amp response, having done that curve, you can calibrate the log amp on a channel by channel basis. And that is simply the way standardization is going to the whole issue of calibration and correction curve for the log amps.

DR. PURVIS: Are they doing that digitally up at the software collection site?

DR. SHAPIRO: Well, I think what happens is that somewhere in the digital processing, they have the correction factor built in. I can't give you the details for that, but I know that they tend to do that.

Otherwise if you are doing digital processing, you will be better off over the range you are going to use. You still want to check the amps.

DR. HOFFMAN: The only thing that I would differently is in the plot, plot the mean channel of, say, the lowest to the highest bead versus the delta, rather than versus --

DR. PURVIS: Sorry. Plot the what?

DR. SHAPIRO: Plot the mean.

DR. HOFFMAN: Then you can see where your actual variation is on your scale.

DR. PURVIS: Okay.

DR. VOGT: So then the lines will no longer be horizontal.

DR. SHAPIRO: No.

DR. MUIRHEAD: No it should be flat if you have been reading it out on your intensity scale, instead of on a --

DR. HULTIN: My assignments are pretty

uniform assignments around a flat line for delta between the peaks. They don't generally go up at an angle like that. They're fairly horizontal.

DR. HOFFMAN: I am wondering why it's falling off so quickly.

DR. HULTIN: Probably where the place is running together and then when it's coming off scale or --

DR. PURVIS: It's not actually running together because the way I gave it, it went back to the previous slide. I am using one of the other detectors holding it constant and getting a region around it and explaining that region into another histogram all by itself. So there is nothing in that region or that population showing up there.

So what you are actually getting into is that there is something going on once you get out to the very end on the FC500 and XL. I will do that in the future, put it on the --

DR. HULTIN: The future will be halfway through the last decade. After that, you have to -- well, you just can't use the quantitation.

DR. VOGT: The other point Norm made but I just want to emphasize this is that you can have properly calibrated standards. And depending

on where you put your standard curve, you might see shifts in the slope because if they fall into one of the bumps or wells along that --

DR. PURVIS: What happens if your residuals are affected.

DR. VOGT: Your residuals are affected. And then imagine what it is like when those standards themselves were calibrated on instruments with some misbehavior of this sort because depending on whether that standard in that batch fell into a bump or a well on that instrument that was used to translate values, that can have the effect of --

DR. HOFFMAN: With what's there -- and that's the delta channel. If you drew a sort of best fit straight line through there, the variation with what you're seeing is what I've seen. Usually it's like plus or minus five channels, which is about five percent. So that is the extreme variation with the mean log amps.

I looked at 40-some calibers coming through the back gate at one point, just comparing, at least as they left the factory, how the log amps were set up, basically doing the same thing, comparing the means or channels of rainbow beads.

I was surprised how consistent they were.

DR. VOGT: And then two operational questions. How far apart should these be ideally? And I forget the second question. First, how far apart should these --

DR. SHAPIRO: A factor of two would be okay, one and a half.

DR. VOGT: So a factor of two would give you about 3.5.

DR. SHAPIRO: You don't want them to be much more than a factor of two.

DR. VOGT: Right. You don't want a real broad range because then you will miss bumps or things in between. Shoot, I forget my last question.

DR. SHAPIRO: For the stereo bands here, you might could say 20 hertz with 20 kilohertz plus or minus half a decibel. That usually sounds like a pretty good spec. But if you do the math, a half a decibel is about six percent. That, in fact, is the variability that you get on the decibel log amps.

So if you try and quantify and you want to quantify to two percent, then six percent could be unacceptable. It sounds great in stereo.

DR. WOOD: At the low end here, the reason that there are variations is that the offsets either from background noise or actual linear offsets in the amplifiers, if you are taking the difference.

The amount of signal that you are getting is asymptotically with an approach someday. So you will at least actually get the XL. It will either flare up or go down based on the offsets that are there at the low end.

DR. VOGT: That is a true non-linearity in measurement, then? Is that right, Jim?

DR. WOOD: No. Actually, the measurement is linear. The amplifiers are fully linear. When you take a log of the magnet that has an offset, the log transformation can't handle the offset. Remember what I showed you before?

DR. VOGT: Right, right, right, right.

DR. WOOD: You end up asymptotically approaching the offset value. Now, if you did it in linear space, all you would have to do is the shift in the lines from the offset. So if you plotted these --

DR. VOGT: As ratios in linear space.

DR. WOOD: -- in linear space, you did

it as ratios in linear space, they would be perfectly straight up and down.

DR. SHAPIRO: I had some curves here that are done on the channel scale and on the voltage scale.

DR. VOGT: So that is a better way to approach the machines that are doing digital processing now, rather than --

DR. WOOD: Right.

DR. VOGT: -- using their --

DR. WOOD: Right, using the linear space, all of your values. Then you will see that the instruments are indeed linear all the way up and down the scale. Your log transformation is what is confusing you because you handled the offset, but if everything was like MX^1 --

DR. MUIRHEAD: So your prediction is that if you plotted this data, instead of taking the delta on the log --

DR. PURVIS: I can take the same data.

DR. MUIRHEAD: -- the ratio and the linear scale, that it would go away?

DR. PURVIS: If what you did is use real linear data, don't take the log numbers --

DR. MUIRHEAD: Re-transformed, yes.

DR. PURVIS: -- not take the log numbers but actually go back to the linear data, what you will get is a line directly, a flat line. The ratio will be the same, go up and down.

And, actually, I take that back. You can't do the ratio because, again, the ratio won't deal with the offset. You actually have to --

DR. MUIRHEAD: You actually have to plot it through the ratio of the slopes?

DR. WOOD: No. You have to actually plot it and show that it is just going straight. You take a particle and show that it gives two lines. If you take two particles and they are going to --

DR. MUIRHEAD: The slopes.

DR. WOOD: The slopes are the same.

DR. MUIRHEAD: It would go

DR. WOOD: What you did is used real linear data?

DR. VOGT: Oh, so a non-parallelism is such a divergent slope, that could be a non-linearity.

DR. WOOD: If you just think of --

DR. PURVIS: If the slopes are the same, then the ratio should also be in a straight

line.

DR. WOOD: No, no. You have got MX plus B1 and MX plus B2. If you take the ratio back, then what will happen is as this goes to zero, you will approach that ratio. And it will do the same thing here.

But if you took these two lines and plotted them, then you should see two lines that are absolutely parallel, with the difference between them being related to your difference in offsets.

DR. HOFFMAN: For two different amplifiers, you mean?

DR. WOOD: That's right. Well, the beads. The beads are different intensities. And this works fine as long as the offsets are minimal because what you are assuming here is that this is all you have to look at. The problem is as this goes to zero, these don't.

DR. VOGT: But, Jim, from the standpoint that the fluorescence intensities are reading on the instrument, then the reading that we get off really does become nonlinear because of the offset, because of the defect.

DR. WOOD: Right.

DR. VOGT: So the bottom line is that --

DR. WOOD: But it's only if you are doing log transforming.

DR. VOGT: But it doesn't work in --

DR. SHAPIRO: It becomes nonlinear on the linear scale, but it's usually --

DR. WOOD: No, no. It's still linear. I can go back and reanalyze the data.

DR. SHAPIRO: Okay. But if you have an offset, you have, let's say, a ten-volt offset in your amplifier, then your signal should be one volt or two volts.

DR. WOOD: Right.

DR. SHAPIRO: If you get a ten-volt offset, 1.1 and 2.1.

DR. PURVIS: Okay. It's linear, but it's not proportional.

DR. SHAPIRO: Right, right.

DR. PURVIS: Okay. What people are assuming is that it should be proportional, which is different from being linear. The upper parts are linear. That is, it's --

DR. SHAPIRO: Yes, but if you are talking about linearity, where you want G2 would be

plus G1 --

DR. PURVIS: That is a requirement to be forced but not linear.

DR. VOGT: Well, you see, that is what I am getting at. I think maybe we are talking about from the standpoint of quantitative flow, we don't want to make measurements in a range where that kind of behavior -- getting back to the practical question, if we did it in linear space with the ratio and we want to see that offset effect occurring, we would not want to use values that were in that range where we started departing from linearity.

DR. WOOD: This is proportional here. It is not proportional here. It is linear all the way across. Linearity and proportionality are two different things. That is, anything that is proportional is liner, but all things that are linear are not proportional.

DR. SHAPIRO: Wait a minute. A linear system is defined as proportional, as a system where the -- a linear system is vaguely defined as a system where the output is proportional to the input.

In the definition, if you talk about

linear systems, you put twice as much in, you get twice as much out. That is the systems definition, engineering definition, of a linear system as far as I can remember. That means that that system is nonlinear.

DR. VOGT: Actually, throughout the whole range, but you just don't see it until you get down to where the offset value is starting to influence your --

DR. SHAPIRO: Yes. I mean, it seems to me that that is sort of intriguing. And the whole simple processing will -- the way you define a linear system, a real linear system, is one with no offset, one where output is proportional to input.

DR. MUIRHEAD: You mean to be linear, you have to go through series is what I am hearing you say. Is that correct? Is that what you are saying? That is what I am hearing you say.

DR. SHAPIRO: Yes.

DR. MUIRHEAD: That is different than what, for instance, I would think of --

DR. SHAPIRO: What we are looking at essentially is -- anyway, those are there where temperature is stabilized. Actually, two of them are from Stanford, and the crappy one is from my

lab.

DR. MARTI: That's this one, the bottom one.

DR. SHAPIRO: That's the crappy one, right. I think I did not identify the manufacturer. The top curve is from a different kind of log amp. There is one kind of log amp that you can make with a single log amplifier. It doesn't have the multiple stages.

You can get a fairly flat response curve on that one. So it gets deviant at the top and at the bottom. It's fairly forgiving, although if you plotted the response curve, it is not the parallel to the horizontal line.

Those modules, I have actually used those modules, have to use those modules in the machines that they built. The company that makes them stopped making them. So I can do that. And, clearly enough, the high-resolution peak detectors that I use replacing my log amps from that company come from the same company cost just about the same.

Meanwhile, most of the commercial sectors use multi-stage log amp modules. That is a PD instrument, that or a BD instrument. I don't

know which one.

So, again, you can see the effect of the multiple staging. But if you've got the curve, it oscillates around the line. So it's pretty stable. Overall, the others and those two are about even.

Now, if you get a single-stage output module to work well, it's tricky. And this is a single-stage output module. It doesn't work well. You can see that they are -- you know, this is not too bad.

Here there is a notch. That notch will translate itself into tieing the little distributions where there should be unimodal distributions if you are at the wrong point on the measurement scale.

So once I discovered and actually sent the thing off to Stanford to get it measured and they said, "Boy, that sucks," you know, once I learned these tricks, I started buying more expensive log amp modules.

But you're still okay if you can find them. I mean, this is easy to do. This basic technique was worked out by I guess Dave Parks and Wayne Moore and Marty at Stanford. They presented

it. It went back to the 1988 ISAC meeting. And it had some antecedents and some stuff that was done in Jonassey's lab.

DR. MARTI: We found slight ones, too.

DR. VOGT: The reason I am asking these questions is people will try to push anything too far. I will, for instance. And what I am trying to get at is how can we define a lower limit, lowest limit of quantification on cytometry?

DR. SHAPIRO: Go look in the first decade.

DR. HOFFMAN: First, you have to say what the acceptable error in your quantitation is. Then you can look at all the contributions to error and figure it out.

DR. VOGT: For the practical end-user, is there a way that you can say doing what Norm is doing here, for instance, I would say that where his curves start to go out of the linear range, if you plot it the way you all are suggesting, I would no longer want quantitative measurements from that point down or that point up.

DR. STETLER-STEVENS: However, in clinical specimens, if you are going to have a range of expressions, you are going to have most

probably who fall in the acceptable range, but some are going to fall in an unacceptable range.

About the only thing you will be able to do, then, is to say it's below the last acceptable range determination, period.

DR. VOGT: Well, you can report the less than or greater than figure or you can adjustment your PMT, recalibrate your instrument, and get a value, which is probably unnecessary.

DR. STETLER-STEVENS: It was a consensus item that we put on the list.

DR. SHAPIRO: On most instruments, you will probably think you are learning between the top half of the bottom decade and the bottom half of the top decade.

DR. HOFFMAN: Good. That's a good rule of thumb, but if you have actually measured your response, then you would have to decide what is your acceptable error in your quantification. If you say it's ten percent, you can go further into the bad part of the log amp. If you say it's two percent, then you can't do the log amp at all. If you say it's five percent, then you work in a fairly large --

DR. SHAPIRO: Okay. But what would

that be --

DR. PURVIS: I'll tell you. We found this. We do quantitation off of this. And we have gotten reliable, reproducible results on the Caliburs that have this type of response.

I just brought this up so that we could see that we need to be aware of it.

DR. SHAPIRO: The curve that I showed represents the way the Calibur runs across the range. And I think that you eschewed things somewhere by using the multiple scale.

DR. PURVIS: And I will replot that so that I can --

DR. MARTI: It won't be scales. Each one of those scans is --

DR. SHAPIRO: It does span the range, but there are some things. Voltage gain is not linear. PMT is not linear with slight voltage. So if he's got a linear voltage scale, he is going to sort of play accordion numbers on this.

DR. WOOD: I think the answer to your question is it depends on the complexity of the analysis that you are willing to put into it. You can remove offsets as long as the offsets are non-random.

If you're dealing with just random noise at the bottom, then you really can't ever get rid of that. But if what you are dealing with is something consistent as an offset, then you can't start to be rid of it.

DR. HOFFMAN: Once you've got the calibration curve of the log amp, basically, I guess Cytomation does this in sort of an invisible way. But, you know, Kathy, you have done this decades ago.

DR. MUIRHEAD: You could cross it over. Nobody wants to do it.

DR. VOGT: Now, again, is that also true of these digital plots, that you could correct the answer from an output of a digital machine by --

DR. SHAPIRO: FC500 is a digital machine. And so is the XL.

DR. WOOD: Right, as long as what you're dealing with is not a random drawing, non-random contributions. You can take those non-random contributions out.

DR. VOGT: And that's what we see on those curves splaying others in the upper right-hand.

DR. WOOD: Right, non-random contributions.

DR. VOGT: Those are consistently shaped because they are non-random.

DR. WOOD: Right.

DR. VOGT: And, therefore, you could make adjustments to that.

DR. WOOD: Right.

DR. VOGT: Or you could say for the practical user because this is probably down in the range of auto-fluorescence and stuff like that. I mean, I am just trying to get a sense here of where we could advise people not to try to do this without doing something special that we don't want to go into here.

DR. SHAPIRO: You don't have to do this with beads. We have used nuclear density pulses, and we have used signal generators on the log amps.

DR. VOGT: Right.

DR. SHAPIRO: The problem is whatever you do on the circuit against the log amp almost always has more offset than we had.

DR. PURVIS: The main point here is you've got to be aware of it.

DR. STETLER-STEVENS: So we say that there is a range you probably should be doing quantitation within. If you've got to go outside of that range, you have to do a whole set of pain in the neck --

DR. SHAPIRO: It's not being a pain in the neck.

DR. STETLER-STEVENS: But it's not going to be done in a clinical lab.

DR. SHAPIRO: It's not going to be done. Look --

DR. STETLER-STEVENS: So you've got to let them know that they have to jump through the hoops to do it.

DR. SHAPIRO: If you need to do this in a clinical procedure, there are people out there who do this. That is to say, first of all, you have the manufacturers. They develop some software. And then you have third party software developers.

The third party software developers were probably ahead of the manufacturers in producing software and reported units and what you do in the quantitation. The third party software users will be out there and developing whatever you

need to calibrate and whatever you need to analyze.

DR. STETLER-STEVENS: At this point, there is a range that you should be doing quantitation in unless you have specific modifications to your --

DR. VOGT: You see, Mary Alice, that doesn't work either. In Lance's data, a lot of his measurements are down in that range because it is a smear from the negative population going out.

DR. STETLER-STEVENS: He does do the extra steps. What I was saying is that we can have as a recommendation that there is an optimal area for doing quantitation unless you go through additional steps.

DR. VOGT: he doesn't really do anything to correct for that. None of us can.

DR. MUIRHEAD: No, but he verifies that he is getting --

DR. HULTIN: That's why the normal distribution is fairly aligned. It's because he has a sloppy measurement down there.

DR. HOFFMAN: That really doesn't matter.

DR. VOGT: So what we're saying is if the median value of a distribution is greater than

the first half decade or so, then you're probably safe.

DR. WOOD: Well, if you just look and say that your noise is in the lower portion of the first decade, --

DR. VOGT: Right.

DR. WOOD: -- then you have to get up into the first part of the second decade. No. If you're at the bottom of the first and you move up a decade, then you're dealing with roughly a ten percent error. If you go up another decade, you're dealing with a one percent.

So based on what your measurement can tolerate, you can now look at even the bottom of the second or the top of the first based on what you can tolerate and then accordingly adjust from there.

DR. PURVIS: Biologic error.

DR. HOFFMAN: Right. Ten percent is a pretty --

DR. SHAPIRO: Also, again, when we do our analysis, it goes from 100 to a million molecules. The first decade is 100 molecules to 1,000 molecules. A hundred molecules most of us find a little --

DR. HOFFMAN: Suspicious?

DR. SHAPIRO: So when you're talking 300 molecules, that begins to be credible. It certainly is at 500 molecules.

DR. MARTI: We've got to move on.

DR. MUIRHEAD: Can I make one point? The big benefit that Norm gets from standardizing the window of analysis. Okay. If you do have a curve that isn't absolutely flat, at least you know you're in the same place on that curve and you're not changing your calibration.

DR. SHAPIRO: There are ways of analyzing this to fix it. And nobody is going to make the clinical labs do it. If it needs to be done, it needs to be done. Somebody will get the job done.

DR. PURVIS: You all are right. I wish I was as smart as you all. If I had moved to place on channel numbers and each one of these curves was lined up on top of one another, it would have been in the same point, instead of looking like it was all over the place.

Quantitative calibration. I am not going to spend a lot of time. It's four peaks, eight peaks, however many peaks you want to have.

Make your evaluation.

We have always or I have always done it off of the log channel numbers. I understand there are some issues with that. Basically, the calibration line that I get as far as the slope intercept, whether I do it off of linear values or the log channel numbers, I am getting the same curve. So I do understand what you're talking about at the low end. So that is true.

So, anyway, you are going to get a slope and intercept. Can we say anything about the threshold? I don't know if we truly can this way, but that was something that was defined before.

Next slide. There you go. There is my regression line.

Next slide. Accuracy, highly dependent on the manufacturers' assignments. This was a problem that we had defined a long time ago, showed that there was a big difference between BD and FCS, Flow Cytometry Standards Corporation. That was resolved in the PE or to some extent resolved. We still have some differences there.

We have relationships that we can use to define and use multiple standards or ways of calibrating our instruments, making sure that what

we have got is right. That is one of the things that we do, compare back over.

A rainbow slope should be the same as my QuantiBRITE slope. If they're not, then something is not right. So that's when I call Nathan up and say, "Look, my assignment don't seem exactly right."

DR. MARTI: What about your MESF slope?

DR. PURVIS: That's what I'm saying. If we are comparing the slope off of rainbow beads --

DR. HOFFMAN: What are you using for your calibration? Why are you using the rainbow beads?

DR. PURVIS: There are numbers that are assigned to that. They are not MESF. I forget exactly how you define your --

DR. WANG: They are quantified on one instrument using actually similar kinds of procedures as what people normally use. And there is just relative intensity between peaks. We need to use a number. We cannot just say a ratio, whatever.

You got actually a cross-particle rate. Rainbow was whatever sets of particles you feel

comfortable with.

DR. PURVIS: The reason I used rainbow was because it's very stable. It's existent. The slope that I get off of that corresponds to a four-decade instrument, which I should have. If I run my PE MESF beads, it should also give me four decades. If it doesn't, then something is not right.

DR. HOFFMAN: I think using the rainbow beads or other hard value assessed to beads like that is great. I use them all the time. As a rule or with a scale, a relative scale, they are great, but the values that are assigned --

DR. PURVIS: I don't use those assignments.

DR. SHAPIRO: What you do is you put the rainbow beads on the same scale as your PE or fluorescein beads. And these are the secondary standards.

DR. HOFFMAN: You can cross-elevate.

DR. SHAPIRO: Yes.

DR. MARTI: The slope should be the same.

DR. PURVIS: And that's all I'm saying. The slope should be the same. I have a way of

verifying that a new set of beads that I get in and I can compare it to the old lot. I can compare it back to the rainbow beads. I can do a lot of things to verify it. What I am getting into the manufacturers is good.

I am having to do a lot of that work, and I wish the manufacturers were doing that work for me so that I didn't have to verify it all the time.

So we had the QuantiBRITES. We had the PE MESF calibrations going.

Quantitative procedures. Do you want me to keep going or do you want me to scan over to the antibody QC?

DR. MARTI: Probably that. Go to the antibody QC? Okay.

DR. PURVIS: Okay. One of the things that we have done is that we go through a complete antibody evaluation. And that's part of my group, Analytical Systems, had done, has been doing now for most of seven years.

The qualifications for all the CD groups that we are using in the clinical lab, some of the CD groups that we get to use in our clinical trials because of the nature, they come to us two

weeks before they want to start the study. I don't have time to get all of my vendors to submit for qualification.

So I tried to go with the vendor that I have had the most success with in getting quality antibodies in the fluorochrome. It depends on the fluorochrome that we are talking about because just because they have good antibodies, if I need to go to FITC, their FITC may not be as good as somebody else's FITC. So we have to make some judgment calls on that occasionally.

We go through and titer all of the antibodies. That's done on a lot by lot basis but also on an order by order basis. So if I have received that same lot in the past, I don't necessarily assume that it is going to have the same performance.

There are all kinds of things that happen during shipment that I need to verify that it is still good. So we do a complete titration on those, multiple donors, multiple samples so that we are looking at the systems that we are actually using.

One of the main things is because we are doing it through one centralized look, I am

standardizing all of my labs. So nobody else gets that choice. I am making that choice for them. I am telling them this is the concentration we are going to use this antibody at and this is how you are going to do your process. I am saying the procedure for them to use.

DR. HULTIN: When your next batch drops and it's half this bright, how will you even notice it on a log scale and you are quantitative with a correction factor?

DR. PURVIS: We're going to talk about that.

DR. FISCHER: Norm, do you buy bulk for all of these different things and re-vial yourself or do you buy them in individual vials and --

DR. PURVIS: Most of everything that we get is in individual vials. I may order 1,000 vials of an antibody so that I have a lot of it on hand. We go through a lot of antibodies. It would be great if I could get them all, say, hey, provided to me in a big 500 ml bottle so that I can do whatever I need to do, but then they have go their labeling considerations, all of their GMP considerations that they have to deal with.

So it's just easier for me to go ahead

and buy in bulk. I have to worry with having that many vials that I have to try.

DR. FISCHER: So do you just track a respective number of vials to make sure that the whole lot that you get in is good?

DR. PURVIS: Multiple vials in those situations. We will open a single vial, randomly choose a vial, and we will mark it as our QC vial, load it up and make sure.

As long as they were received in one container, I am going to make the assumption that they all went through the same conditions. If I get them in different shipments, each shipment is assigned a separate tracking number. We label each of the vials with the tracking number. One of those vials will be assigned to QC, and we will do a titration on that.

DR. VOGT: And, Norm, when you titrate, do you titrate to try to get the plateau?

DR. PURVIS: Looking for saturation. You did your qualification. It was primarily to look at intensities, quality of the antibody. Was it signal to noise ratio, what kind of labeling of the positive cells, what my background levels were? Is it being provided to me in saturating

concentrations? A number of factors go into that evaluation.

So I am doing a direct comparison of each vendor's antibody on the same sample processed by the same person all in the same day. So I am removing as many of the processing variables as I can.

So if we get in six different vendors for CD11B, we will get three samples. They will do a five-point, two-focal serial dilution for each one of those antibodies on three donors And it will all be processed through the same procedures and running the same instrument at the same time. And I can get a direct comparison to see whose antibody has the best performance.

And it's not just mine. I will consult. If it's an antigen system that I am not very familiar with, of course, I have to consult with people who have more experience with that. So there are a number of things we have to look at.

DR. VOGT: Do you often see something like PE20? We were talking about it yesterday where you just cannot get saturation.

DR. PURVIS: What I will typically see -- I think I have not a good one from one that

doesn't give a great saturation. Some of the antibodies that as you do the titration here, you are starting it neat and then titering it out twofolds or 1 to 2, 1, 4, 1, 8, 1, 16, keep on going. What we will see is a slope coming down. And then, all of a sudden, there is a change that will occur.

This here I think is something to do with either nonspecific binding or a low-affinity binding site that someone is continuing to bind with the higher-concentration antibodies.

DR. MARTI: What happens if you five percent positive parallel for that?

DR. PURVIS: Oh, it's a straight line. As part of that, we look at the signal to noise ratio. If it is truly a background issue and I have a negative population, then what we will see on the signal to noise is we will do something that will be like this. Okay?

Can everybody in the back see that? Essentially all I am doing is plotting C20. I've got non-b-cells, lymphocytes. And I've got the b-cells. The negative non-b-cells, probably too much antibody being in there, they will have an increased background.

As soon as I start removing some of that, the background will go down to whatever their auto-fluorescence levels are if you're not having t-binding while the b-cells remain at the upper intensity levels.

Once I start going below saturation on them or this breakdown, then they will start dropping off on a drastic basis. And it's like a bell-shaped curve.

Typically this is what I am looking for. So this is the concentration I am going to use it at.

DR. LENKEI: What we were talking yesterday was that we would start with the other procedure. We would start with that, and you go up. The problem is that it never stops. It goes up and up and up. So the problem is that you can choose --

DR. PURVIS: But I'm also looking at the signal to noise. I am also getting a high background on my other cells. So my opinion is that if I am seeing this, optimizing my signal to noise, as long as my optimization on my signal to noise is in this area, not over here in this area, then I have a good antibody that I can use.

DR. LENKEI: Yes.

DR. PURVIS: I can get reliable quantitation off of that antibody. I can get consistent staining off of that antibody.

DR. DAVIS: Do you worry about FC binding? I mean, that may be the --

DR. PURVIS: That's one of the things that here recently, I have gotten a number of different lots of antibodies: CD3 FITC, CD8 FITC. I've seen some binding that didn't make any sense. So yes, there is FC that will get involved.

We see this in most -- we know that the side guys have a receptor that gets picked up that will stain on monocytes and neutrophils. That is now being bought to some extent in some of the additives that are being put in there.

FITC, though, I have seen here recently. And if I go back with the CD64, CD16 unlabeled, stain the cells with that, and then stain with that, I am blocking it. So I am blocking a classic FC receptor type of binding.

So if I see that, then that is something also I don't want to have. So here lately we have had to change our vendor because we were getting a lot of FC almost 2 antibodies.

DR. FISCHER: I don't see the problem with CD cells. We get very little back. We have very little FC body on that.

DR. DAVIS: Yes.

DR. FISCHER: It's mostly the 16.

DR. DAVIS: It's small numbers. That's why. But it might explain some of this randomness everybody is concerned about.

DR. PURVIS: I see what you're saying. So it will go in and block the FC receptor and see if this flattens out some?

DR. DAVIS: Yes.

DR. PURVIS: That's a good experiment. I haven't tried that.

DR. HULTIN: I can see that same thing on 45RO titrated it. You get these twofold maximums because you are under-saturated. And then you go beyond there, and then you double it and just keep it consistent. What else can you do?

DR. FISCHER: I think the signal to noise that you pointed out is the biggest thing because I can get a huge increase in the amount of signal, but if I am also getting a huge increase in the amount of noise, I mean, I gain nothing in the long run. I might as well start with PMT.

DR. PURVIS: And many times I don't get the bell-shaped curve on my signal to noise. What I will get is a very flat line, kind of like what you would expect from a saturation curve that then falls off once I get below my saturation.

So, again, that kind of indicates that this is just a background staining issue or a very low-affinity binding site that somehow causes problems.

DR. MARTI: For lack of a better definition, it's nonspecific binding. And if you did, in addition, a reaction where you put in gold, that's an expensive way to decide where that point is, but that would be another way.

DR. PURVIS: So that's classically how we have approached our antibodies.

Go to the next slide. I'm sorry. This all has the --

DR. HULTIN: Do you do this titration with whole blood or a fixed number of PBMC?

DR. PURVIS: It's done with whole blood. It's done with bone marrow. It's done with cell lines. It depends on what the system is that we are working with and what the antigen is that we are going to be trying to test. It's going to be

on tissues and bone marrow. So I am going to put those into the process so that I am titering it on the appropriate --

DR. HULTIN: In whole blood, do you fix the number of lymphocytes, for instance, or do you just throw in these 100 microwaves you're saying by throwing whole blood in?

DR. PURVIS: I understand what you are getting at. What we typically will do with, say, a CD20 is you have a very low percentage of b-cells and whole blood. I will go back with rainbow cells or a different cell line that has a very high expression that I can then hold my cell number to a million cells per tube, and I will see what is going on in the whole blood, look at what is going on in the cell line, which if I can keep the cell line at a saturating level and I don't have problems in the whole blood with nonspecific binding or background issues, then that is what I am going to go with.

Your point is well-taken because --

DR. MARTI: How would you control it? And if you go to PDLs, you are going to have --

DR. HULTIN: Well, just for a titration, I prefer a fixed number of PDNC. And

then, of course, everything has got to work in its optimal place for whole blood because that reflects the patient. And that is just a philosophy.

DR. MARTI: Have you ever compared whole blood to high-tech --

DR. HULTIN: For what purpose?

DR. MARTI: Well, the fluorescence intensity.

DR. HULTIN: Oh, absolutely.

DR. MARTI: And are they the same?

DR. HULTIN: No. But you have got a decision on how to make a titration. If you want, how are you going to use the same amount of blood each time? It's just something I've --

DR. MARTI: So you are going to determine the saturation or ideal concentration on PDLs but then apply it to whole blood?

DR. HULTIN: Verify it on whole blood and then optimize the staining for time and temperature, et cetera. And then you just go with it.

DR. PURVIS: In the leukemia lymphomas, though, one of the things that we have to watch out for is a number of times, the pathological sample that we get is going to be primarily tumor.

So it may have 90 percent b-cells there. We're using a million cells, and they are all b-cells. If I haven't appropriately titered my antibody to accommodate that high number, then my intensity is going to be much less than what I --

DR. MARTI: Has anybody ever seen published data on a human tumor, live human tumor, cell line and decreasing concentration of the cells? Has it ever made a damn bit of difference of any antibody? Going once, going twice.

DR. VOGT: I was about to ask the same thing.

DR. MARTI: We don't say that. If you have something that has 100,000 count, do you take 100 microliters, 150, or do you take 20 or do you take 10?

DR. LENKEI: Can see the bone marrow.

DR. HULTIN: Keep the volume. I mean, you're asking a question. To me, the volume and the concentration, the effective concentration, in the antibodies are what I want to keep the most consistent after I have picked out my share of antibody.

DR. DAVIS: But when I was publishing, I mean, that was one of the required ways of also

making sure we would be higher than the saturation if you had enough. Eventually you are going to absorb.

DR. VOGT: We've never seen samples like you have all seen, but we did see some CLL lights, some PL counts in the few tens of thousands. I was never able to demonstrate any diminution of staining caused by that compared to the normal 200,000 t-cells.

It's my impression in the whole blood that most of the antibody gets sucked up in wherever. I don't know where but neutrophils, red cells, wherever.

DR. HOFFMAN: A lot of antibody does not go anywhere.

DR. PURVIS: You can see in the background staining on that cell that you don't expect it to be on.

DR. VOGT: Right. I mean, basically it seems to me that in general, we are in huge excess in a whole blood trap and for specific binding because we have got such nonspecific sinks for the antibody

DR. PURVIS: A good example in the United States is for PNH, 55/59. Are you going to

stain whole blood? Are you going to stain isolated cells? What are you going to stain there?

DR. MARTI: Which stem are you going to analyze?

DR. PURVIS: Well, there is literature that says that both are important to take a look at it.

DR. MARTI: You said the lymphocyte is worth --

DR. PURVIS: Oh, I don't. We will put a co-stain in for identifying those. We put in the 55/59. You could put the glych 4na in for the red blood cell. If you are looking at red blood, you can have it just based on scattering.

The idea here is if you do a titration optimized on isolated cells and then your technician goes and stains, putting whole blood in there, guess what. You're not going to see 55/59. So there's a number of things that you have to consider here as to how you are going to approach your staining, whether you are going to soak it up.

Kappa lambda is another example. If you don't wash away the plasma, putting the kappa or lambda antibody, guess what. You're not going to see any b-cell staining.

Those are the types of things that you just have to keep in mind.

DR. ORFAO: I think we are coming back to a point that came yesterday. We need to know the bio yield, the molecule. It's like a liter or two microglobulin. You have to look not only at how much the cells are expressing the protein with b-cells but how much volume you also have to do from how much soluble protein will be there.

DR. FISCHER: That's like a twist between the wash, no wash, lyse wash, no wash. It becomes a major factor. A plot of b-cell markers that we work with, it was at least a sign. So they just blocked the receptors, blocked the markers with b-cells.

So you pede them out with huge amounts of antibody if you're not or you have to extensively watch the cells beforehand. And when we remove the nonspecific error, actually specific binding, but the non-antibody binding, to the specific marker you are interested in. We haven't found a good antibody that goes around that yet.

DR. PURVIS: Okay. So we do this for all of the antibodies that we get in. So we did the extensive vendor quals. That is not something

that you want to just keep on doing. That takes a lot of time, a lot of effort.

And so we want to stay with our primary vendors at that point until we see a problem develop with that antibody. And I may say, "Hey, we've got a problem here. I'll contact the vendor."

They'll go up there and say, "Yes," they can identify it. They're resubmit their antibodies to me. If not, then I'll have the vendors, other vendors, resubmit to me and see if anything has changed.

Vendors do improve their antibodies with time. They also let them degrade with time depending on how long the culture has been going that they are working from. It may have moved and lost some of its affinity. So those are things that you have to keep in mind.

For a while there, every antibody that we got in that was FITC or PE, we determined the F to P ratio. We weren't doing the quantitation of these antibodies. It was just another quality control measurement that as an engineer, I went overboard, but it was nice to follow because it did show that you do get differences in F to P from lot

to lot, that if you are going to do quantitation, you have to be able to account for long-term studies, when you are going to be using multiple lots of antibodies, how that F to P changes.

So now we only do this with the antibodies that we are truly glued to do quantitation with. There are times when my clinicians will come to me and say, "Hey, you titered this antibody out.

Actually, when we hand new products off to them because we combine all of our antibodies together and hand them a cocktail that they're going to use, we'll make a six-month batch of cocktail. They do a comparison to the previous lot that we have made.

Even though we have already done the QC release, they go ahead and do it to verify that we haven't messed up or missed something. And they will see a change in intensity between the old lot of a cocktail and the new lot of a cocktail. And they will call us up and say, "What is going on here? Why do we have this change? Have you changed the titration? What is going on?"

Previously, for the FITC and PE ones, I can go back and say, "No. This was truly a change

in the antibody source that we got. We had an F to P change." Now most of the time I can say that that is what it is, but there are times when we have to go back and do some checks to see if we made a mistake. So there is a need for that.

Should an end-user have to determine the F to P ratio? No. I think it is way too difficult. Abe used to like me because I bought a bunch of his Quantum simply cellulars all the time, but it is very difficult.

It is not an easy process to go through. You have to titer your antibodies onto the beads. You have to do a timed analysis so you end up with 42 tubes that you are setting up here so that you can get one measurement that will work. And then you are making a decision on whether the data that you generated is giving you a good F to P. Anyway, we go through a number of qualitative and quantitative assays.

Next slide. Reagent cocktail formulations. We have already gone through this. Standardization. And I still have Cytometry Associates. Boy, my boss would get mad at me for that. We're in Esoterix, by the way.

That yellow doesn't show up very well.

Sorry about that.

DR. VOGT: Oh, I am glad it is there. I was worried I was hallucinating.

DR. PURVIS: And there's also a font that I am using that isn't on your computer. So it won't display some of the mathematical symbols here. But essentially Jim has already shown you some of the equations up here that we use. I must have had a duplicate in here. Like I said, I brought in a bunch of slides.

Here is one antibody evaluation that was done for a clinical trial. I can go to a different slide if you all want to see some of the considerations that went into that clinical trial.

Here is CD11A. Initially the idea here was we wanted to look at both total and free sites. So I needed two epitopes. I needed an epitope that was the same as what the drug was going after as well as epitope that was not blocked by the presence of the drug.

Initially we were going to try to do it off the MESF. Two vendors, both in FITC. We went through a number of extensive qualifications to find out which antibodies of this once we reduced it down because I don't want to show you all of the

different antibodies that we looked at.

We did calculate F to P's. You can see that vendor D, vendor F, there are some differences in the F to P's on the antibodies. This titration is also -- we got a saturation level. You can see that it is beginning to go through a break-off point. So how would I choose this? I would want to work up in this back up here as long as my signal to noise wasn't a problem.

You will also notice if I am comparing MESFs, we have got some PE up here as well. There is a big difference in the scales. How am I going to compare the binding in MESFs? I can't compare FITC to PE.

I really am going to have a hard time comparing this red and this blue -- those are two FITCs; those are these top two up here -- and being able to really state anything quantitative, do some real calculations for this clinical trial.

But if I use the F to P ratios that I have calculated here divided, the MESFs by the F to P, that kind of agreeance there is wonderful. This isn't the only system this works in. We have done it in multiple systems this way.

So antibodies, even though they are

different clones, as long as the epitopes aren't biologically masked in some way, I should be able to quantitatively get the same antibody-binding capacity as long as I have a good F to P ratio if I am using quality antibodies and if I do my job right to begin with.

Next slide. Tandems. We have already talked about that. Again, I don't know if this has any more. I apologize. I don't even know what slides we are coming into now.

Multiple antibodies against the desired antigen. This is what we were just talking about. If we are doing one of these studies, I have got to do my titrations of the antibodies first in the absence of drugs, see if there are saturatables, see how the antibody is going to react with no drug around.

And I have to go back and do my same titrations in the presence of different levels of drug to make sure that the antibody is going to behave the way it should with those drugs on board.

Is it going to mark the total, no matter how much antibody or drug is prevalent, or can I prevent any of my antibody from binding if I am looking at an epitope-sensitive location? That

is part of what we have to go through. Again, those are just restating the same thing.

Antibody evaluations. This is on the Quantum simply cellular. This is typically what you would see. Essentially the F to P ratio, we are getting four measures of the F to P ratios with these four peaks. Okay?

I know the a, b, c, the binding capacity of that bead. Each one of those peaks, I get MESFs, divide each of them by its binding capacity. Now I have four MESF to bead or protein or probe, I guess, is the better word to use here.

DR. VOGT: It's hard selling that one, Norm. It never seems to grow legs.

DR. PURVIS: Anyway, we do four of them. We take an average. Well, guess what. This is how I can use this to tell me whether I am getting a good F to P because if I have a high CV in these four measurements, something is not right here and I have got to go back and take a look at it.

So we go ahead. I really need to know what my F to P is. We do a titer onto the beads. I have four levels showing my titration. In most cases, when they converge and you get all of them

having good agreeance, then that is your true F to P ratio. And that is how we determined it.

Next one. I'll show you good and bad. This is just how we do it.

I think that's it. I don't think we really need to. So here are the important issues. You've got to have absolute agreeance, then the lower limits.

My opinion is many of the therapies that we are going after now, you are not going to give this therapy if the person is not showing CD20 or whatever the antigen is that you are trying to block.

In most cases, what we are going to be quantitating is for the higher levels outside of that second decade. What is the expression? Let's look at the disease states. Look at the literature. Which ones have responded and determine what ranges of expression we might want to give the drugs or therapies.

The activation markers is where the lower limits of quantitation actually come in. That is something that is probably for a different discussion. I know it is an important one.

I used to do platelets. I used to look

at platelet activation. But it is a decision that you will have to discuss at a later point. Our instrumentation is getting us there now. We are getting better and better optimal collections. So I will stop unless you want to see some of the actual --

DR. MARTI: Norman, if you were to take our sites and examples of antigens that you have made quantitatively, CD4, CD8, CD45, what is the variation on that? It doesn't have to be any that I just cited.

DR. PURVIS: Well, one of those slides that I had to go on through showed CD4, CD5, and CD19. If you are looking only at MESF, you have some huge deviations that we were seeing there. Part of it was due to the beads themselves at the time.

MESF assignments would be off. Abe can address this. At one time, our PE calibration was really high. We made an adjustment to that, which caused a step change. Even without that, I was having some variation in the bead assignments, but they were pretty minimal. So I was having problems.

As long as my F to P was determined off

of the same set of beads and I accounted for whatever my calibration was, it tightens things up. And I will see on the order of five to ten percent variance in the population, which is great. There are other ones that are much higher than that that may be 20 to 50 percent. It just depends on the antigen systems.

Some of these are going to be -- a number of them that we are going to want to look at are going to work okay as long as we standardize the use, consistent protocol. And we always use this. We may have some biases if somebody else wants to use a different methodology, but statisticians may be able to tell us how we can correct for those bias measures.

DR. LENKEI: Speaking about one of the antibodies, what is it to test the F to P ratio in others who are not that good? How do you account for that fact?

DR. PURVIS: Can you pull up my other presentation? Repeat yourself. I'm sorry. I was playing with the computer.

DR. LENKEI: You are speaking about antibodies good to measure the fluorescein to protein ratio, and others were not that good. I

have said the same thing. For some antibodies, you can get agreement; for others, not that good. So how do you --

DR. PURVIS: I really haven't had -- there are some antibodies that from a particular vendor, I cannot get a good F to P. There may be something in their soup or whatever. Maybe there's 3 PE or whatever that is messing things up.

But for the most part, I can go to a different vendor and get a good antibody from that, get a good F to P. So I haven't really come into cases --

DR. LENKEI: So you don't know the reason. You have not --

DR. PURVIS: I have questions.

DR. MUIRHEAD: You're defining that as agreement with the ratio obtained for each of those four peaks. So how big is the standard deviation?

DR. PURVIS: You saw my blanks. My blanks had disappeared with that one histogram They weren't there anymore. Where did they go? They had found antibody. And it shifted up into my first peak.

What happened? What is going on there? There is nothing good. I mean, it shouldn't be

binding anything. So my antibody was somehow binding the beads.

DR. VOGT: Where is it?

DR. PURVIS: This is it.

DR. VOGT: This is it?

DR. PURVIS: Yes.

DR. VOGT: Which slide is it on?

DR. PURVIS: If you all want to see this thing, I can go through that one assay and give you a couple of considerations. If you don't, then I will sit down and shut up, and we can go on with the rest of what Mary Alice had.

DR. STETLER-STEVENS: Actually, we have a snack break. We are going to finish early today. So you can have a late lunch and have a snack right now. We can take a break now and have something to eat, go to the bathroom, and then come back.

(Whereupon, at 12:22 p.m., the foregoing matter was recessed for lunch, to reconvene at 1:00 p.m. the same day.)

A-F-T-E-R-N-O-O-N S-E-S-S-I-O-N

(1:05 p.m.)

DR. STETLER-STEVENS: We have got a lot to accomplish, and we have a short period of time. Alberto has got some additional comments to make about compensation. We want to hear from him. Then we will move into our action items.

DR. ORFAO: Actually, I had no additional comments. I have just to summarize from where there is clear agreement. I think that there is clear agreement that you need multi-color because biology is like that. So you need multi-color.

I would just like to add a small example of that. You might need quantitation of more than one protein in these things. This is a paper recently published in *Blood* showing that when you were using anti-CD20, at least *in vitro*, it was only given CLL cells if the number of CD9 complement regulatory molecules was below a certain length. So then you need 20 and 59 for the same cell.

So multi-color might even be necessary to measure 2 different proteins simultaneously. I think all of the issues that you pointed, where you

have to have a total protein and a certain epitope of that protein, they also talk about quantifying two different proteins simultaneously.

So the second issue -- I think it is also clear -- is that data should be stored without manipulation. So you should store the regional data, so not already compensated data. So this I think is also a very clear message and I think a very clear consensus on at least what I understood.

DR. VOGT: As you may have masked over in that regard, with the older instruments, are you suggesting that people turn compensation off?

DR. ORFAO: What I am suggesting is, let's say, that at least thinking of what you would propose would be what people would be using. From now on, at least they have to think about this.

DR. PURVIS: I will play devil's advocate and tone that point up. Not everybody is going to be a one-cytometer site and --

DR. MARTI: How about when appropriate with future instruments?

DR. PURVIS: Well, what I would like to see happen is like what Coulter is now doing, where you get 20-bit linear data files automatically saved down with your compensated data as well. If

there is a compensation problem, you have the ability to go back and --

DR. MARTI: How about data stored with and without it?

DR. SHAPIRO: Well, if you have the matrix, if you've got the high-resolution linear data, then you could store the compensated data because if you have a matrix, you can --

DR. D'HAUTCOURT: I will add something. If you don't have the capability to store the data in linear mode in high resolution, I suggest you make the compensation during the acquisition and not after.

DR. SHAPIRO: Yes, pretty much.

DR. D'HAUTCOURT: If you need to transform the data in linear and come back, that increases the problem.

DR. SHAPIRO: That's true. And if you have --

DR. D'HAUTCOURT: So if you don't have the new machine, don't use the technique of non-compensated data.

DR. FISCHER: I think that without compensation is going to have to apply to those of us who have been fortunate enough to have the newer

age instruments.

But I have the older one, too. And that data all gets saved compensated because there is no way around it. I mean, really, you can turn the compensations off after you have checked it to make sure that all the PMTs and everything were going to balance out.

But we all know that occasionally with the PMTs, you want to make sure you are going to be able to compensate all of the data, especially with the multiple-level fluorochromes that we have.

DR. STETLER-STEVENS: How about the statement that it is optimal to have it stored as uncompensated data with the appropriate, that you have to have the appropriate machine?

DR. SHAPIRO: Well, that is high-resolution data.

DR. STETLER-STEVENS: Yes. So there's an optimum system.

DR. SHAPIRO: Yes.

DR. STETLER-STEVENS: I think we have reached consensus. That is optimal. However, if you can't do that, you have to compensate the best you can and realize that compensation is a problem.

DR. WOOD: Another thing we could do is

store under-compensated data; that is, actually go ahead and do some of the compensation. And then you could apply a correction to it.

You won't see as much artifacts of the compensation if you have already done a fair portion of it already. Typically you are only dealing with just a very small change to go from being under-compensated to being compensated.

So if you are always under-compensated, then you can correct it. If you are overcompensated, you can't bring it back.

DR. VOGT: Right. So, actually, part of that is don't overcompensate. That would be number one. Whatever you do, don't overcompensate.

DR. SHAPIRO: If you overcompensate, then you risk not being able to get it back.

DR. VOGT: Right, right.

DR. SHAPIRO: I don't know that we have to --

DR. STETLER-STEVENS: Maybe we should --

DR. SHAPIRO: -- micromanage that. I don't think we have to micromanage this because this comes up, and this really relates to a

situation -- it is clearly better to have the newer machine with the digital processing, where you could shift back and forth from a linear log with log amplifiers and their deviations from ideal response, don't come into the picture. It is clearly better to have that.

As Bob Hoffman pointed out, well, you know if you have a four-color FACSCalibur and you use fluorescein and PE and PerCP and APC, then essentially the hardware compensation on that instrument is adequate to the task and you can do --

DR. STETLER-STEVENS: Not all of it --

DR. HOFFMAN: Are we expecting that people in clinical labs are going to set up their own? Are we trying to tell people how to set up their own quantitative assays or are we assuming that somebody knowledgeable is going to set up a quantitative assay system that people are going to use?

DR. STETLER-STEVENS: I would never assume that somebody knowledgeable is going to set up assays that people are going to use in clinical laboratories.

DR. HOFFMAN: You would not?

DR. STETLER-STEVENS: No.

DR. SHAPIRO: Well, I think the point is that --

DR. FISCHER: The clinical people have spoken.

DR. SHAPIRO: They have spoken. The point is that going back to the process model of CD4 counting, the knowledgeable people got together and got their act together before they defined guidelines for everybody else.

If we envision following more or less the same process, then, in fact, the first successful attempts -- well, the first attempts at quantitative flow have already been made. They have been made in sophisticated laboratories.

And the next few attempts will probably be made in sophisticated laboratories, at which point we can start to figure out whether we can, in fact, export this stuff down to the level of laboratories with less sophisticated instrumentation. When you are doing less sophisticated instrumentation on less sophisticated users, it may be that you would have to transfer the procedures.

DR. HOFFMAN: Or that you just work up

procedures that are robust and tolerate the --

DR. SHAPIRO: Well, yes, but the question is who is going to need to do these how many times. When we start out, the likelihood is that for clinical trial purposes, you are not just going to go to every lab in the States to do the quantitative analysis. You are going to have some --

DR. STETLER-STEVENS: Bruce's test is one that may be done in every hospital. Sepsis is a problem.

DR. HOFFMAN: Right, but Bruce has developed a robust system.

DR. STETLER-STEVENS: And what about juvenile diabetes, which is a problem, or Type 1 diabetes? If we start to get into more and more applications, you are going to have to think eventually they will be moving down.

And I think that stating an optimal method and caveats of what you have to be aware of for compensation --

DR. SHAPIRO: Well, that's true, but I think that a lot of that is going to be ad hoc. There are some methods you are going to be using --

DR. FISCHER: You will be able to apply

guidance. You will not be able to apply specifics.

DR. VOGT: Two things on that. One is that if we said that ideally data would be taken at high resolution on compensation, that may give people the opportunity to buy new instruments because you do need a reason to buy a new instrument in most cases, some justification.

The second thing is -- no, but I think that is important. We have got to keep the instruments being sold to keep this community together.

And the other thing is -- well, to me that is all kind of related, that you can't --

DR. MARTI: I can see that you people would be very useful in helping us write a three-part justification for sole source --

DR. VOGT: For sole source. Right. And the second thing is that the NCCLS H42, I think it is, the original guideline that was developed by the committee that Alan Landay chaired, is now up for renewal, up for review and refinement and reissue.

My boss, who is a part of that, has put me as a liaison on that committee. So I will be following along with that. He would like to

discuss that at the CCS meeting. Mary Alice and we are talking about how to do that. So this is stuff that can be --

DR. DAVIS: I chair that.

DR. VOGT: Oh, I'm sorry. Yes. Bruce is in charge of that. That's right.

DR. DAVIS: More accurately stated, I am looking for somebody else to do the work.

DR. MARTI: It sounds like a good definition of a chair.

DR. VOGT: Bruce, is it fair to say that these are some of the things that could be considered in that meeting?

DR. DAVIS: Oh, yes. In fact, we are hoping to add like CD34 counting and --

DR. VOGT: Right.

DR. LAMB: We're the folks who wrote the CD34 counting. It's a very small document. It was deemed by the committee to be too controversial because I mentioned the Ice Age method along with the Milan method. Then they threw it at me.

DR. MARTI: Kathy?

DR. MUIRHEAD: Can I just put a big plug in for that process because I think a lot of what I am hearing from Mary Alice is possible

within that context. That process goes through not telling people what the optimized method is because there is no optimized method for every assay you want to learn, but highlighting what are the issues that have to be considered and controlled, what example are there out there of things that have worked or not worked in a given context, provide references and examples, and then gives people a process to go through in terms of identifying whether they got it from the manufacturer or whether they put it together in their own lab, whether they have an optimized process. I think that is an extremely good model.

DR. STETLER-STEVENS: I think that if we do believe that there is an optimal method for one specific question, there is one thing that is the best way for compensated or uncompensated data, if we feel that there is a best way, we shouldn't be afraid to say what the best way is along with the caveat you can do it by other ways, but you have to be really careful and good about it.

So there are optimal methods. And sometimes you just do what you can. But when there is something that is best --

DR. SHAPIRO: Of course, if you want to

do quantitative flow cytometry, that implies that you can't get the right answers if you don't have correctly compensated data.

DR. MARTI: Some may even question they're not sure whether you can get the answer if it's properly compensated.

DR. WOOD: Still along the idea of this micromanaging point of view, has anyone had an encounter with a legal system getting involved here? That is, if we start giving too many nooks in specifications, then standard of care issues will start coming up.

And if there is a misdiagnosis or mistreatment based on a test, then what are they going to define as being standard of analysis and so forth, consolidation sorts, things of that sort?

So those are issues that once we get very specific will probably come back and haunt you.

DR. STETLER-STEVENS: But if you just put out a document saying, "Be careful" --

DR. SHAPIRO: You all be careful now, you hear?

DR. STETLER-STEVENS: You said nothing.

DR. WOOD: That is what I am saying.

DR. STETLER-STEVENS: So you have got to have a balance between them. If you just said, "Be careful" or "This stuff is hard. So do it well," I would do it well.

DR. HOFFMAN: For compensate, for a compensation reference, one, two, three, whatever --

DR. STETLER-STEVENS: Norm, you --

DR. PURVIS: I was just going to comment that I think that compensation that we keep bringing up here depends on the system that you are compensating. If we are talking about something that is right out of the third decade, fourth decade, compensation that may be a little bit off is not going to cause any problem.

I don't have a problem at all working with compensated data because most of the systems that we are going to be compensating, that is going to result in a very minute error compared to just the biologics in the system it will measure.

I think if you want to set some of these qualifications, then you set it on the low-end quantitation areas. These other ones I think we are probably beating something to death that doesn't need it.

DR. STETLER-STEVENS: So compensation when you are dealing with low intensity, for instance, you're saying that you have two different sets of sort of rules.

In most cases, it's not a problem as long as it's done appropriately. Hardware, software, whatever, you can do it appropriately. When we have a certain set of circumstances, the compensation becomes an important issue. And it may be extremely difficult to achieve accurate compensation with hardware compensation. And this may impact upon the values. That would be an unusual circumstance, but --

DR. PURVIS: At that time, you are going to be choosing something that you don't have to worry about compensation. Either you are going to be setting up your system so that the other four prongs are not going to impact, influence your results by a foregone combination choice. I think that is --

DR. VOGT: I think that's one of the useful things that is on here as you design your analytical cocktails.

DR. STETLER-STEVENS: But when you are dealing with kappa and lambda and being positive or

negative and you're comparing your two twos, I mean, you do have an impact in some neoplastic systems. The way you set it up, like, for example, I see a difference in values in the tube where the light chain is positive and the light chain is negative. I know it is compensation. It has got to be.

DR. PURVIS: So the choice there, where you put in quantitating PE that is being influenced by your FITC, then you quantitate on your FITC, where you have got your kappa and lambda.

DR. STETLER-STEVENS: Yes. Only it's pretty low intensity, and I can't use FITC. So there are going to be areas where it may be a problem, but it is not going to be in general a big problem.

DR. PURVIS: I don't think so. I think in most cases, you can choose the proper fluorochrome combinations so that what we keep discussing here is not going to be as many of an influence or as much of a --

DR. HOFFMAN: That would just be one of the guideline checklists.

DR. SHAPIRO: The problem is how many assays are we talking about here. In other words,

we're really talking about doing things one at a time. So there are some very general things you can say about quantitative flow cytometry in general, clinical and otherwise.

As you get toward the specific applications, then protocols are going to have to be defined. Whether or not there is a form of guideline, NCCLS may decide it's not worthwhile to issue a guideline for a test that is going to be done 500 times a year.

If people are very interested in the particular disease that is going to require that that test be done 500 or 1,000 times a year, they are going to get together and design a protocol.

Anybody who is really interesting in following that is probably going to become aware of the existence of that protocol and want to follow it. That's when you get into the ad hoc stuff.

Otherwise, things are too open-ended. We are telling everybody everything. We are telling people more than they need to know. Until you get down to some of the specifics of the biology, it is really hard to define what you need in the way of the technology to solve the problem because some stuff is easy.

DR. ORFAO: Well, I think that, further, the FITC related to compensation, I think there is also consensus that we need to do FITCs to do compensation. I think at least in this area, there are some types of facts that there is I think agreement.

The first is that it should match with your fluorochrome combination exactly. That way your proposal was that you actually had your same antibodies staining beads.

DR. PURVIS: Or cells.

DR. ORFAO: Or cells.

DR. PURVIS: So if you have 20 different antibodies, we are going to have 20 different compensation sets.

DR. HOFFMAN: No. This wouldn't be 20 different ones. You can't have the fluorochromes.

DR. PURVIS: Yes. I agree with you. Again, even with the tandems, if you are going from one vendor in most cases, you can get reliable compensations.

When you go across vendors, then that causes problems, and you need to be very aware of it. And within some of the vendors, you have to be aware that from a CD group to another CD group,

it's required compensation.

For FITC PE, some of these common antibodies --

DR. MUIRHEAD: You have to verify through your reagent sets that that assumption that if I run the compensation setup with this pair of antibodies and this set of antibodies and then I am actually running my test samples with a different set, that I am not going to bias the result if you want to do quantitation.

Now, I mean, I would argue that like you just did a minute ago, which is minimize any of the compensation problems you can up front by your reagent choice and don't get into it. If you don't have to compensate, don't.

DR. ORFAO: The second thing I think there is agreement that they should be bright and I would say that probably should be important to have at least two different intensities, which should be in the positive branch, not like negatives and strong --

DR. VOGT: Three people.

DR. MARTI: And by that, you wouldn't accept a negative bead or unstained cell.

DR. HOFFMAN: Is that for confirming

compensation or for --

DR. ORFAO: For setting.

DR. HOFFMAN: Compensation, where basically you are measuring the mean, mean, median, whatever you want, of the spillover of FITC and the channel FL2 and the channel FL3, channel FL4, whatever.

If you put two populations in there, now you are measuring the mean of the population or you can measure it because basically what the mathematical matrix compensations come up with is what percentage or based on what is a percentage of green fluorescence from fluorescein is appearing in channel 7. It needs a number. It is easier to get that number with a single bright population than with multiple bright populations.

DR. SCHWARTZ: But can it do it?

DR. VOGT: You would want that to be in your linear range of measurement, right? You wouldn't want something so bright that it was nearly against the upper boundary. So can we --

DR. PURVIS: The system can't handle it.

DR. VOGT: Right. So can we say something in the third decade? Is that --

DR. HOFFMAN: What I'm saying is that if you're doing -- I don't like this terminology -- visual compensation, then having multiple populations is being useful if you are confirming compensation that has been set by some methodology. Otherwise, then multiple populations can be useful as long as you are not looking for the quadrant so that all of the compensated values are below a quadrant because that is mathematically correct.

DR. VOGT: As long as you are not trying to do what people try to do --

DR. HOFFMAN: There will be patterns you can see that you get used to where the compensation will be below a quadrant and the higher values will see this population. I just want to make a general statement that for correct compensation, we're going to need multiple compilations.

DR. MARTI: I think I can see a problem if you had two peaks. Would you get two peaks spilling over into, say, the PE channel or would it come as --

DR. HOFFMAN: Yes.

DR. MARTI: So, then, that must just drive that algorithm insane.

DR. SCHWARTZ: It wouldn't because the higher one is going to --

DR. MARTI: Dominate. I see.

DR. PURVIS: That depends on how much spillover there is and whether it's statistically --

DR. SCHWARTZ: But it will be proportionally the same. It has to be.

DR. HOFFMAN: You will pick one peak or the other.

DR. MARTI: No. Pick them all.

DR. HOFFMAN: It is going to depend on which approach you are using for compensation.

DR. ORFAO: So the last I would say two points regarding compensation. I think it is also the compensation is a true ultimatum.

DR. FISCHER: You would like that, actually, more. It's not on the -- you really like to have it automated because it would certainly make it a little more standardized for the folks so they wouldn't have to come in and necessarily mess around with setting.

And then it would eliminate the word about whether the data you were setting was properly compensated if the compensation, automatic

compensation, worked correctly every time.

DR. ORFAO: And the final issue is the issue of validation. You need a biological sum.

DR. FISCHER: Yes, no argument there.

DR. VOGT: That validation, again, might not necessarily be that it falls below your horizontal data. What is the validation of good compensation, I guess, to put it the other way?

DR. D'HAUTCOURT: I will say for the validation, I find the double population very important. Not for the compensation but for the validation, it's more. If we have more than one population, it's different intensities for what it is.

DR. ORFAO: For the compensation?

DR. D'HAUTCOURT: I mean that in only one is enough.

DR. MARTI: With the individual ones that you used for the compensation?

DR. D'HAUTCOURT: Don't use line. It's the reason why we use automatic compensation. We don't use line or display of something. It's --

DR. MARTI: The single cell or B that you stain for each compensation control for each fluorochrome --

DR. D'HAUTCOURT: For each fluorochrome.

DR. MARTI: For the mixture after you got done running the individuals, if you put the mixture together, would that satisfy validation?

DR. D'HAUTCOURT: No.

DR. MUIRHEAD: Not if he wants multiple labeling of the cells.

DR. MARTI: Okay. That means you just have to devise another control in your system.

DR. HOFFMAN: The other thing is that using cells in the auto-fluorescence of whatever you are using as your "negative" is not the same as the auto-fluorescence of your stain population.

DR. VOGT: Right, right.

DR. HOFFMAN: I mean, your t-cells and b-cells will be using CD3, whatever, for your stain population. You know, do the t-cells have to be the same auto-fluorescence t-cells?

DR. VOGT: No, they don't. No, I think not.

DR. HOFFMAN: So, basically using CD45, which is saying all white cells, and you have to have another auto-fluorescence sample, at least doing something so that you're comparing the

auto-fluorescence of the stain sample with the same population.

DR. MARTI: Would it be best, Bob, under those conditions to have an unstained sample that contained all populations represented?

DR. HOFFMAN: That's the ideal.

DR. MARTI: So that might be a useful control, a completely unstained population.

DR. HOFFMAN: That happens in CD45 and does in multiple populations.

DR. MARTI: Yes. So, then, if you ran a CD45 and all your fluorochromes of interest in an unstained tube, then --

DR. HOFFMAN: That would be your positive control.

DR. VOGT: Those would be your --

DR. ORFAO: You get more staining for 45 on your auto-fluorescence population.

DR. VOGT: Why would that be --

DR. ORFAO: Because you have the sites strong enough for 45. It would be less auto-fluorescence for the neutrophils.

DR. MARTI: Randy, what's your nine-color positive control?

DR. FISCHER: For which channel?

DR. MARTI: You find the single stained cells for b for each of the nine channels. Now you want to demonstrate that it is working properly. You are going to run the unstained mixture and you --

DR. FISCHER: I never run unstained.

DR. MARTI: Okay. What is your positive control so that if it runs properly, you will have confidence in your test?

DR. FISCHER: I generally run one that has monocyte, b-cell, t-cell markers, and nK markers all in the same tube. Sixteen, 33, 56, 4, 8, 3, 19, 20, and I don't know how many that is. I'm missing --

DR. MARTI: Your positive control, the complete immunophenotyping in one tube, and then anything else that we wanted to examine. If we didn't feel we could do that one right, then we had little hope of doing anything else right. So I guess that's what you mean by the positive control, rather than just mixing individuals back together.

DR. HOFFMAN: About the control, you are getting expected results. As long as you are getting expected results, I guess that is -- I was thinking maybe more about using the biological

cells in the purest sense and being able to check if the set is correctly, in which case you would measure the median of all of the cells.

DR. FISCHER: If I were going to do that, I would pick one antigen and do all the different colors.

DR. MARTI: But they just said I couldn't do that. I mean, that is the same thigh.

DR. FISCHER: That is basic. I would still do that because that is the only way you are going to have any idea because, number one, you can make sure all of your percentages are going to be the same.

DR. MARTI: Well, okay. Let's --

DR. FISCHER: You have got to have sort kind of uniformity across here because you have the standard because you are going to measure the entire qualification of the instrument.

DR. MARTI: What is the difference, then, between CD45 labeled in all nine colors? You stain each one of those individually. So you are going to have a CD45 FITC, a CD45 PE, and then you mix those together after you have run them individually. Is that what you are accepting as your positive control or is it that you used those

nine reagents --

DR. FISCHER: I would argue that something similar in Mario's remote controls works the best for fluorescence minus one. But I don't necessarily follow this procedure.

DR. MARTI: I can tell you that in six colors, we don't really see any difference in terms of automated compensation if we use one antibody with several different fluorochromes or if we use the individual antibodies that are in our test panel. We are not good enough to see a difference if there is a difference.

DR. FISCHER: I have never seen a difference in any of the four, six, or nine-color stuff that I have done where I have set everything on known cells that I have been using for God knows how long with these antibodies that I have been using.

The changes in my compensation from day to day and my PMT settings from day to day are so small that if you track them over time -- we're talking a long time now -- for most of my other instruments, not science. I've just had that about a month.

But for those instruments, if you were

to look at that, you would plus or minus the error of the machine probably for being able to detect those things. There is just not a large, significant change. And even after we have our routine PMs done, for (a) repairs, if the equipment is put back in the condition that the manufacturer wants it in, I can call up my last seven spot.

It almost matches perfectly with running a sample I have just stained so that the changes are so minimal to where known antibodies -- and that is the problem, of course, that you have got to use known antibodies and cells that you are used to working with.

DR. SCHWARTZ: If they're so small, why make them?

DR. FISCHER: That would be the argument, but I do that just as a matter of history. Well, I also like to have the evidence that yes, I actually did do this. So there was nothing that also went wrong with the instrument because somebody came in in the middle of the night and a lot of people, rather than have this problem.

We have people who work almost 24 hours in our lab running instruments. They come in. And we get some people who run some of the damnedest

things in the world. They can only run one before they have to clean the instrument because cells begin to clog it up.

Well, that could make a difference if they don't do a good enough job. We all know what happens when somebody leaves their instrument or leaves any instrument not in the best shape. It can affect your results dramatically. So that is why we run --

DR. LENKEI: It's not like those in such a -- instrument and use them as they want because then it's really a problem.

DR. WOOD: In terms of automation, this was occurring to me. If people used at one other point; that is, a double positive point, you could make sure that you had a full rectangular figure in there because even though with explaining it out, you have no idea of when you have gone necessarily too far, guess when you have gone too far.

If you have that extra point up there, then your populations will line up. And you will see the curvature in the data.

DR. MARTI: Would you maybe, say, do stained cells with CD45 FITC and PE in the same tube so that you would get 50/50 staining?

DR. WOOD: Well, especially the first thing that popped up in my mind was back when we had the CD4, CD8 sitting right up there. You could actually with that system make a perfect rectangle. If we had the perfect rectangle, everything would evolve a little, the set limits. You would actually see curvature like that.

DR. MARTI: I guess that comes back to designing your system.

DR. HOFFMAN: Right.

DR. MARTI: If you can design that in, that sounds like it might not be a bad idea. Okay. Let's say that we have finished, beaten to death compensation. I think we would now like to -- oh, I'm sorry.

DR. HOUTZ: Just thinking about what you said, compensation guidelines, it seems like with analog versus digital, with maybe a new algorithm with a bioexponential display, that perhaps make some recommendation and then cite references. I just think that being overly detailed is not going to be able to adequately cover everything for their purposes.

DR. MARTI: I am going to tend to agree with you. So what is left are some action items.

We have listed them as protocol, phycoerythrin problem, education, applications, and then looking adequately to try to discuss what consensus we have arrived at.

Now, for protocols, we talked about what are the things that you need to look for, what is this list of items, this checklist. Some of those things, as I recall, are temperature, length of incubation, the lyse, and the lyse to wash.

DR. SCHWARTZ: Fixation.

DR. MARTI: Fixation would be the fourth.

DR. SCHWARTZ: Concentration of antibodies.

DR. SHAPIRO: In terms of the protocol definition, we were talking about particular things in a sense, CD20, where we don't do saturation. Is the C20 database -- and we have other things on the database on the Web.

So it seems to me that, particularly if you are looking at quantitation of CD antigens, there is data there that can be folded in and should be folded in to the design of any protocols because that will give us the maximum information about what we are looking for and how we might want

to look for it.

DR. SHAPIRO: What is that? There are protein reviews on the Web, which basically keeps data on analog CD and other things. And so for somebody to cite, "Gee, we ought to be quantifying this antigen" and have --

DR. FISCHER: Is that through NCDI? Yes, it's NCDI. Okay.

DR. STETLER-STEVENS: I think on these items, if we have people who are willing to head a subgroup to do this, we should give them rein to do it the way they are going to do it and report back and not say, "I want you to do this, but I want to tell you what you have got to do." Bring it back, and we can go over it.

We did get an aide who has committed to work on it and to get others to help do the work, not to do all of the work, but to sort of spearhead this effort.

So what you need to look at, the other thing is how do you optimize. What are a set of instruments of how you can go about doing this. So we are not just saying you have to specifically direct x, y, or z, but how do you determine if it is x or if it is y.

And then there was a lot of discussion about doing a study to validate these concerns are real. We will leave them to determine the study that they want to do, but many people have indicated that they are willing to participate. Some people have told me that they want to participate in this study. They think it is important.

DR. LENKEI: I think it should not be too complicated. It should be a first stage. We get some information which is possible to be statistically analyzed and proceed.

DR. SCHWARTZ: Understood.

DR. LENKEI: Understood.

DR. MARTI: Lance, you suggested a focus protocol.

DR. HULTIN: No. You were talking about CD20s. I offered that. I would just give it a time, this time and temperature stuff. I will send the data for distribution. You can review it and see how it goes because you couldn't quite decide all the factors. I'll just give it a shot, and we will see where it goes.

DR. VOGT: The other thing, remember, for that group I think is that CD38 is both an

important problem for you all as matotologists and a problem that has been largely solved.

So all that you need to do there -- not all, but you need to draw the link between the solution to this problem in the HIV world and its application in the b-cell malignancy world. That is probably the shortest drive you have. That is probably the par three on the course.

DR. MARTI: I would agree with that. Move on to the --

DR. STETLER-STEVENS: Next is the beads.

DR. MARTI: An attempt to make some kind of -- I may state this wrong because I don't understand the problem, but getting some kind of agreement between the F to P ratio on PE conjugates. How would you define that problem?

DR. VOGT: I was suggesting that we have a smaller group together and look at all three -- I hope I have it right -- of the aspects. One is the MESF microbead standards so that we can have MESF units that are comparable. That would involve looking at the ones that are currently available, looking at their spectral characteristics, which we can get Dolph to do, and that sort of thing.

The second component is the conjugates and looking at, again, spectral characteristics of the conjugates, the immunochemical characteristics of the conjugates. By that I mean their molar F:P ratio and their binding qualities.

And then the third thing would be to do an actual laboratory exercise with a send-out, with calibrators. folded down into that would be things like could an Alexa dye serve as a stable surrogate for assigning MESF units across different types phycoerythrin preps.

DR. SCHWARTZ: The problem is you can't because that excitation specter is not the same. PE is excited by a lot of us at 488 and by Howard at 535.

DR. SHAPIRO: Right, right.

DR. VOGT: I think those are things to be discussed. We would like to move toward a PE MESF particle that could be used to standardize any PE conjugate, but that may not be possible. So that is one of the conclusions we reach.

DR. MARTI: I'm just simple-minded here. Is it possible that Alexa could be used as a soluble fluorochrome to standardize a biological fluorochrome?

DR. SCHWARTZ: Yes.

DR. VOGT: Well, that's what I said, and you said no.

DR. MARTI: My PE --

DR. SCHWARTZ: You said you were going to use Alexa to assign PE beads, not Alexa beads.

DR. VOGT: Well, I don't know. What I said was use Alexa as a stable surrogate standard bead. That's all I said.

DR. SCHWARTZ: The answer is no because the excitation specters are different. So on different instruments using different --

DR. VOGT: It says the variation. I am not talking about the detail part.

DR. MARTI: Can't you use a solution of the fluorochrome, of the appropriate Alexa fluorochrome.

DR. SCHWARTZ: For?

DR. MARTI: To assign MESF units to PE.

DR. SCHWARTZ: No.

DR. HOFFMAN: As long as they are PE units.

DR. SCHWARTZ: No because if you are excited with two different lasers --

DR. HOFFMAN: You can say, you know,

for the excitation --

DR. SCHWARTZ: You can do that, but then you might make everything in fluorescein MESF units for each --

DR. SHAPIRO: I am not saying that is a good idea. I am just saying it is possible.

DR. SCHWARTZ: Oh, it is possible, but I agree. It's not --

DR. MARTI: PE, if you think it is complex on a bead or on a cell, I get the impression that it is pretty complex in the solution itself.

DR. SHAPIRO: Well, it is pretty complex in the solution itself, but the point is that solutions are a great way of assigning massive units to low molecular weight guides. And they are probably not the most wonderful or appropriate methods for assigning units to, in fact, doing proteins or cantons or, for that matter, GFP. You can't use the same tool all the time. I mean, it's --

DR. MARTI: You can use fluorescein to assign MESF to a particle. Why can't you use a fluorochrome --

DR. SHAPIRO: It's still fluorescein on

a particle. Yes, if you have got auto-fluorescence in the fluorescein channel, you can record that auto-fluorescence and fluorescein message units, even though it's not from fluorescein.

So fluorescein goes into the PE spectrum. So, as Abe said, you could use fluorescein to assign PE, to measure PE fluorescein message units, but it's not accomplishing the same end.

DR. SCHWARTZ: So what if you said, "Why don't we make Alexa beads and Alexa-labeled antibodies?" Terrific.

DR. VOGT: Well, that would be one, but it's another issue, though.

DR. SCHWARTZ: If you want something in that area of the spectrum, that would be the way to do it, not cross-fluorochromes so you can get confused.

DR. SHAPIRO: Yes. But the point is that there are other ways. I think the BD process in producing the QuantiBRITE beads and reagents has produced an acceptable, if not a NIST-traceable, standard with PE.

DR. VOGT: Right. And that would be certainly the starting point and also the Bangs

beads to look at what are the PE standards we have got and how close are we and then what do we need to do to close whatever gap exists.

DR. SHAPIRO: It was my recollection that I have not seen a process way down in the process that the Bangs would be analogous to the published process to the BD beads. Maybe I missed it.

Presumably if the steps are laid out, if there is a discrepancy, then if there are two processes, both of which are completely defined out in the open and then you come up with different numbers, then I worry about it.

If there is a process that gets defined for one of the products but not for the others yet and then there is a discrepancy, then let's define the process for the second product. That is not something that we should worry about.

DR. SCHWARTZ: We need to deal with the process of assignment of MESFs, the beads, not worry about the one-to-one antibody stuff yet.

DR. SHAPIRO: Right.

DR. SCHWARTZ: They are two different things. Let's take care of the beads first and make sure --

DR. SHAPIRO: Yes.

DR. SCHWARTZ: -- if you get a PE bead, you get the same answer, no matter what your subject, just like you have the CD4 labeling and for the most part, everybody who makes a CD4 labels about the same level of 50,000.

DR. PURVIS: I think that the main difference there is coming from sources. It's actually, even though it is RP --

DR. SCHWARTZ: This when we came up when we had the problem about I was way off on the PEs on, all of a sudden, the batch was way off. It was that I was assigning against a derivative of PE, which is a disaster. When I got a whole bunch of those lots of that derivative, I had an 80 percent CD or PE from six different sources. My variation was only eight percent.

DR. HOFFMAN: This wasn't in a paper. I didn't do it. Looking at lots of different PEs, it was like a 30 percent.

DR. VOGT: It was 30 percent, yes. That was what --

DR. SCHWARTZ: But if you took out the BD one, it drops. I am not saying that -- I mean, it is just different because you were saying PE one

is much better, much purer, much more control, which I believe.

But it doesn't agree with the other ones. When I saw the data, it was like that. And then the PE one was way up here. I also got the same problem when I started choosing a blank bead. I went. I had 20 lots. I picked my blankest one, and I ran out of it. It was very embarrassing after that.

DR. HOFFMAN: But there are the same issues with PE or any other fluorochrome. You need a standard, and you find a solution of a certain purity.

DR. SCHWARTZ: All of these things need to be addressed in this --

DR. STETLER-STEVENS: Who is going to help you?

DR. MARTI: I still think you can explore the issue. Part of the subcommittee would have the prerogative to explore whether or not the appropriate Alexa dye could be used as a surrogate.

DR. D'HAUTCOURT: As a surrogate --

DR. LENKEI: I think for this thing to stand up, which is not kept there and it's passed to some bank or other, how is your traceability for

this?

DR. VOGT: In order for them to call something an NTRM, there is a process they have to go through in showing how their values relate to what was originally traceable and how that trace is continuous from the lot.

That is a very good question, I think, and I don't know the answer. That was a very vague answer, you might have noticed, but I am presuming they cannot create something they call mistraceable without having a protocol to trace it.

DR. SCHWARTZ: But they will trace it back from NIST. When that runs out, there is nothing traceable.

DR. VOGT: Well, there has to be. There has to be some process. We can't --

DR. SCHWARTZ: That needs to get back that same kind of thing or have NIST do it.

DR. VOGT: Right. It may be necessary from time to time for NIST to make another RM. Remember, Dolph is not intending even to try to make an SRM. So he is already dumbing it down a level in order to keep from having to go to the Vatican to get a value assigned.

DR. SCHWARTZ: But they don't make any

of it. They get it from Molecular Probes or Bangs, whatever. They just characterize it. That is all they do.

DR. VOGT: I think one of the first questions is, certainly the first question is, the available PE standards are. How do they agree? What are the sources of disagreement? How is their spectrum matching? Nobody knows that.

How does the spectrum match on the beads compare to the spectra of the conjugates? Nobody knows that. So there are a number of easy questions for us to answer.

Actually, all we have to do is wait for Dolph. So the answer to the question is me. It doesn't matter whether I am there or not, but Dolph is the most important.

DR. FISCHER: I think you have to throw Molecular Probe as a main source of a lot of these fluorochromes in there --

DR. VOGT: Right.

DR. FISCHER: -- because if you do intend eventually on bringing in any of the Alexa dyes, they obviously have to be in on it because I know BD and some other people are licensing some of these, but at the same time, they are the main

source of beads.

DR. VOGT: Right. So the other people who are on this group -- and I have already talked to them -- are Yu-Zhong from Molecular Probes, Yu-Zhong Zhang; and Jorge Quintana from Coulter, who is eager to do this; and Bob or his designated alternate contact at BD; and Nathan at Bangs. And then I wanted to suck Norm Purvis into this, too.

So, as I said, we will take an arm and a leg of Norm. You all can have the other arm and leg for your protocol group. And we will return some fingers and toes.

DR. PURVIS: We may be only giving fingers, though.

DR. STETLER-STEVENS: Maybe you have something in your group you could sacrifice.

DR. MARTI: Okay. Moving on. Education. I wanted to just throw out a comment. Somewhere along here in planning for the scene in November, on a couple of occasions, we have talked about having some kind of quantitative tutorial the day before that meeting. That may be tacking too much onto that meeting because there is going to be a day after the meeting, really, to continue this meeting.

DR. STETLER-STEVENS: Before the meeting, we have the course. Then the day right before the meeting, we are going to have regulatory affairs people meeting and also --

DR. VOGT: We hope to piggyback. I will talk to Bill Caldwell, who is running that. Now, is that the day before the meeting?

DR. STETLER-STEVENS: Well, the meeting starts at night, Coulter lecture on Sunday night. So Sunday.

DR. VOGT: Sunday is the meeting.

DR. STETLER-STEVENS: It's regulatory affairs and the other.

DR. VOGT: Okay. Okay. So then the NCC unless Ligand Immunology Committee would meet on that Sunday date.

DR. STETLER-STEVENS: Then we have the meeting. Then we have quantitative flow follow-up meeting. So it --

DR. VOGT: Yes. I would agree with you tacking is too much.

DR. MARTI: Okay. Well, any other suggestions?

DR. VOGT: Oh, on PE, I'm sorry. This is staring right out. I wanted Lance also because

we don't need as many fingers and toes from him, but as a person who can speak most for how you do this stuff, he should have been right after Dolph.

DR. MARTI: Does anybody else have any comments on education?

DR. STETLER-STEVENS: Randy had comments, Howard had comments, and Bob had comments. So we put them on --

DR. SHAPIRO: So if we are devising educational materials, what is the venue for --

DR. STETLER-STEVENS: I think coming up with what needs to be presented in education --

DR. SCHWARTZ: You need to put Jerry on it.

DR. STETLER-STEVENS: Jerry?

DR. SCHWARTZ: He has been doing it for six, seven years.

DR. STETLER-STEVENS: We can sort of liaison with I'll get Brent Wood on it, who is head of education committee for CCS. I will liaison with these people with ideas of we need to really approach this. And, for example, we could have -- the only thing I can think of is he would probably be happy to have part of the flow course.

They have special lectures the last

half day, which they have had topics that are not -- immunophenotype leukemia is a big one or minimal disease, a big one. We have huge topics. There are little topics like myelodysplastic syndrome, immune studies in HIV.

It doesn't apply to everyone, but there are important things that could have, for example, a class about an hour long lecture from one of the astute group to go into some of the issues we think need to be addressed.

We have to decide not just quantitative flow. You're talking about things that have got to be standing that lead to an understanding of the quantitative flow. So you're talking about the crawling stage and then the walking stage. So what are the principles you feel are not taught well that allow one to understand to do quantitative flow?

I can talk to Brent about this and try to get this in as a special lecture, for example.

DR. MARTI: So you're saying that -- what is it, the Saturday before the CCS meeting?

DR. SHAPIRO: What about the dusk to dawn?

DR. STETLER-STEVENS: This meeting is

packed. We have a leukemia, lymphoma section that is about two hours. We are having a computer room set up with cases because people want a lot of case work.

But I think that the course work -- and there is also some luncheon work. We have this time about eight luncheon workshops, but that is something that is kind of long in the process for that, but we could have a special topic session as one place.

This is people are coming because they need to learn about flow. The flow course is hugely popular. There is a waiting list always. We give a date that you have to apply by, and we close a couple of months, a month or two, earlier because it is full. Then there are people who say, "I will pay my money. Put me down first for the next one."

So this is a good place to teach people. So let's identify what they need to be taught to have that --

DR. HOUTZ: I would like to be involved in that process, actually, but I'm thinking in terms of like a lesson plan, like let's try to organize exactly the --

DR. MUIRHEAD: Is this a basic fluorescence limitation? I mean, I am still trying to understand what you want.

DR. STETLER-STEVENS: Well, the --

DR. VOGT: What I said originally was --

DR. STETLER-STEVENS: If you could do it in an hour.

DR. VOGT: -- that if you began with the premise that it is all quantitative flow, then, instead of teaching quantitative flow --

DR. SCHWARTZ: Flow is quantitative.

DR. VOGT: Flow is quantitative, right.

DR. MARTI: Whether you know it or not.

DR. VOGT: Right. Flow am ready. Mary Alice, which flow course, now, did you mean?

DR. STETLER-STEVENS: It's the flow courses attached to the Clinical Cytometry Society meeting.

DR. VOGT: Okay.

DR. MARTI: I think the way I would suggest that we leave that is that I --

DR. VOGT: Abe and I have done this the last couple of years together. He had been doing it for some time. He and I have done this the last

couple of years at Bowden. I think that is a different thing to --

DR. STETLER-STEVENS: It's a different group.

DR. VOGT: Right.

DR. MARTI: I think what you are saying is that this is an opportunity to --

DR. STETLER-STEVENS: If you guys can come up with multiple places to present, first you have to come up with what do you need to teach, what do you feel people do not know, especially clinical laboratories. Then we find places we can do it. Okay?

DR. MARTI: We have that already outlined.

DR. STETLER-STEVENS: Okay.

DR. MARTI: We could maybe scrunch it down, but I think the way to approach this is to slowly integrate into the CCS course --

DR. STETLER-STEVENS: Or other places. There are many places to define what you have talked about, what is missing in education. Does everybody need to know quantitative, having to do quantitative flow and use beads? No. Everybody should understand the basics.

DR. VOGT: But I think those are inexorably entwined. You can't go in and see a pattern on a set of quantitative beads and understand what it is telling you, then you don't know what your flow cytometer is doing.

DR. STETLER-STEVENS: Right. But you don't need to sit down and discuss do you want to determine MESF values, do you want to do this. Do you know what I mean?

DR. VOGT: I agree.

DR. STETLER-STEVENS: So this group is to determine the fundamental knowledge that is lacking in general.

DR. VOGT: Why don't we chart it, Mary Alice? I was as loud as anyone about this, I guess. I don't know about one hour, but I would say a maximum two-hour introductory level, what fluorescence measurements in flow cytometry.

DR. MUIRHEAD: The Jim Gill lecture is one.

DR. SHAPIRO: What is it, now?

DR. MUIRHEAD: The Jim Gill lecture.

DR. SHAPIRO: Well, where's Jim?

DR. MUIRHEAD: I don't know where he is these days.

DR. STETLER-STEVENS: So you guys come up with this. And then it's a matter of targeting places to do it. Others can help you do that, but you need to come up. This is a --

DR. VOGT: But what I would say about that I think is it would be very helpful to have him involved in this. The place where this could really have an impact is if we came up with something that each of the companies would present in an hour of the time, of the week's time, that they have for training so that everyone who was trained on a flow cytometer would get the same message about this, then that would probably change the world.

DR. STETLER-STEVENS: Not everybody gets trained on a flow cytometer.

DR. VOGT: Those we can pick up later or at the same time or something, but --

DR. STETLER-STEVENS: Somebody gets trained when you buy it. Then afterwards, they train other people. And other people come in and they learn. So that is why continuing education is important.

What we need to convey -- and then we have to target where it goes --

DR. VOGT: But, you see, the other --

DR. LENKEI: What I wanted to say is that, for example, they have a lot of courses for the ethics courses and current events courses. So I think it should be a collaboration with the companies, with Coulter, with Becton Dickinson, and to see exactly how these courses are because I know they have a very good activity in place.

DR. STETLER-STEVENS: Yes. One site is not going to solve the problem. So that's why it is targeting multiple areas. And you know what? We don't have to tell you how to do it because there is --

DR. DAVIS: We might put a CD together and convince the world.

DR. STETLER-STEVENS: Yes.

DR. HOUTZ: I think a lot of it, too, involves identifying standards. What is a standard, really, in flow?

DR. VOGT: Well, those we have. I mean, those things are adversity counters.

DR. STETLER-STEVENS: A CD actually is not a bad idea. Phil McCoy has put together a CD that he gives to fellows on basic flow cytometry principles.

And you can yap at them all day long. Give them a CD. Some people English is a second language. And you talk to them. And they don't know what they're getting. But they're going to take the CD. They look at it, and they play with it. And it is helpful. So it's not actually a bad idea.

I've got a copy of it, and I give it to our key path scholars. So to have them to sit down and -- you know, this is the Game Boy age group coming into fellowships and residencies. And they would much rather do that than read a book.

DR. VOGT: Oh, that's good news. Oh, great. This is after a lifetime of '80s music? Then they come in to --

DR. STETLER-STEVENS: Okay.

DR. SCHWARTZ: Future brain surgeons.

DR. SHAPIRO: The surgery is all done by remote control now. It's the Game Boy generation there, too. You can take out a gallbladder in Paris now. Of course, now we --

DR. FISCHER: The problem is they went in for a hernia operation.

DR. HOUTZ: Educational issues. To the group as a whole, we do offer workshops on

quantitation tools in flow cytometry. We use QuantiBRITE beads. It's a wet workshop. We actually go through and we use CD4 and we use CD38.

We have been doing that for about a year now. I developed it, and I teach it both here in Mansfield, Massachusetts as well as in California. So, I mean, I could certainly talk with our trainers as far as how we could implement an hour's worth in a week.

I think it's a great idea to make sure that we are aware of this particular aspect of flow cytometry. There is a lot of information here that I think is fairly fundamental that we have been talking about for flow cytometry, but I think that without properly defining standards, calibrators, controls, and so on, I think people just aren't going to really understand what quantitative flow cytometry is.

DR. VOGT: Do you have a good marketplace for that course? People have been --

DR. HOUTZ: I would like to see more of a turnout, but we have had about 15 people attend.

DR. FISCHER: I didn't realize you guys were offering that. The education of the community as to its availability is not --

DR. HOUTZ: Well, it's been on the Web and --

DR. STETLER-STEVENS: And another thing --

DR. HOUTZ: -- our newsletter. It is advertised in our newsletter.

DR. STETLER-STEVENS: Randy brought up this to have local groups, low flow users' groups. And it may be you talking to Burt and saying about how to bring that to local groups and have people come in and give talks.

DR. SHAPIRO: And the Boston group. Well, there is the Boston users' group. And then there is a group called EBG, the Boston users' group, flow cytometry group, which is a subset of applicants together for happy hour every month. From that group, everybody wants to have a big workshop on compensation, which we are now organizing. That could probably be readily extended over to quantitative flow.

We get people. We certainly get people from as far down as New York for some of our bigger meetings. So that may be a fairly big shindig. That is probably going to be sometime in early June because they wanted me to do some of that. Then I

have to go to New Jersey. Then I have to go to Los Alamos. But it looks as if we are going to have something cooked up, something happening in early June.

DR. FISCHER: The Chesapeake group does some of the stuff. We are going to do another one of our joint ones with the newer group. Again, it is probably going to be in the fall this year. That would certainly be a workshop.

Now, last time we had one of these meetings, even though we sent repeated requests to Becton Dickinson to do a workshop, they didn't submit their payment for the thing and, in fact, didn't even tell us they wanted to do a workshop until we sent out the first copy of the itinerary. And then they said, "Where are we on there?"

And we said, "Well, what did you send us to do?" So it will be a case of I will make sure Burt gets the first notice on that.

DR. STETLER-STEVENS: We need to have people who are interested in it from the industry to have --

DR. FISCHER: We had offers from the companies to do this. We only had like 150 people at this meeting, but if you reach 150 of the flow

people in this area, you're doing a pretty good job.

DR. STETLER-STEVENS: We have an education group, another group to write up the applications of quantitative flow lab work because our findings and what we write up and what we do has already been accepted for publication.

So anything we do is not wasted here, even from a professional point of view, we are going to get some benefits. And we are going to reach people. Larry has volunteered to spear this meeting, people to help him come up with applications.

DR. LAMB: Fine. I got a couple of people I want to draft. Okay. I want to draft Burt because he runs around the country and talks to people who ask him what they can do with quantitative flow because he runs the workshops.

And I think, to balance the stick, we will probably have to have another manufacturer as well that has a lot of interest in that instrument and reagent manufacturer, but I'll work on that.

The second one I want is Jerry because Jerry can answer some regulatory concerns with applications. And I think that that would be

helpful. Do we have anybody out there in the HLA community that we liaison with who you might suggest, somebody who is not here but might respond to a draft?

Think of Brey. Brey is over-committed at times about --

DR. SCHWARTZ: What about Dan Cook?

DR. LAMB: Okay. I will talk to him.

DR. MARTI: Is Dan Cook in Chicago or --

DR. SCHWARTZ: He's in Cleveland.

DR. MARTI: Cleveland. Okay.

DR. LAMB: Yes. Brey is pretty much a point person for all of flow and all of HLA and was the president of Oxygen last year. Actually, this year he is charged with the responsibility of planning the meeting. So he is out of pocket a lot.

I think a couple of manufacturers, one regulatory person, and then I reserve the right to tap Bob's work in diabetes or tap Bruce's work with CD64 or to tap somebody like Brent Wood or Mary Alice for some leukemia ideas to help us compile a document that would start simple and work its way up to more complex.

DR. FISCHER: Mary Alice, could I ask you to put something else on there since Larry brought this up, the regulatory concern. I mean, there has got to be something done for the regulatory concerns in this whole thing. What is going to be the government's role in all of this?

DR. LAMB: See, that's why I picked Jerry for this committee because I think that before the nose wheel can even go up, we have got to have some of those ideas.

DR. STETLER-STEVENS: Also, there is a list who is really spearheading a lot in the CCS regulatory committee and is working in that area to help.

DR. FISCHER: Because we all know that if the FDA issues a paper and says, "When you do quantitative flow, you must use this bead to set your machine up and you must use this bead to do this or you must use this system to do that," everybody out there has to do it or they're not in compliance with the FDA. And, therefore, a lot of their data is considered not in compliance. And, therefore, it can't be used for drug discovery or treatment protocols.

DR. SCHWARTZ: Or reimbursement.

DR. FISCHER: Or reimbursement.

DR. MUIRHEAD: That's why you're much better off to do it through things like NCCLS documents because Jerry has already said --

DR. FISCHER: Well, what I am saying is --

DR. MUIRHEAD: So is there going to be don't get --

DR. FISCHER: We want to know in advance. Now, if some of the things we're doing may end up running afoul of something that the FDA would look at and say, "Oh, well, that's a place we should control," rather than a place that we want to keep them out of.

DR. MARTI: Even if there is a NCCLS guideline or even if an FDA guideline, the Congress permits you by law to use another procedure. We just have to show that it's equivalent or better. You have to validate it.

Yes?

DR. STETLER-STEVENS: Can I go back to education? I think we should include Jean-Luc or Alberto in education because we don't want to just think --

DR. VOGT: In English.

DR. STETLER-STEVENS: Yes. So unless I hear --

DR. VOGT: That's also --

DR. STETLER-STEVENS: -- "No, I won't," I am going to put their names down.

DR. VOGT: Sure. And that is also true of B. I mean, I thought of that, actually, just as I moved on to C, but we don't want to do anything unilaterally in flow cytometry. I don't have anything to do with the State Department. So also --

DR. STETLER-STEVENS: We need to ask some people. David Barnett and Jann are also interested in being a part.

DR. VOGT: Sure.

DR. STETLER-STEVENS: But they couldn't come. They had another meeting.

DR. VOGT: Right. In absentia here.

DR. STETLER-STEVENS: And Phillippe.

DR. LAMB: I'm going to see Jann next month. He is in my session at the CCS meeting. So I will talk with him a little bit about application as well.

I wanted to go back to one question. I think that it is a question that I asked yesterday

and sort of has still been grinding in my mind, which also says I am probably going to be tapping you as well, Lance, for some information.

As I said yesterday talking about application, the CD4, CD38 system is probably the best defined system out there for quantitative flow and it's simple. So I asked a question.

I think it's one of the things the application needs to answer is why do people not write orders for it. Why is there not a system developed for it? The answer is, well, we don't know if we are going to get paid for it or something.

So if we are talking about an application where all of this wonderful engineering and technology that has been discussed is going to actually work out there in the clinical arena, some physician at the end of the line has got to make a decision that this is critical for patient care decisions or, else, it is better than something else as far as the economics are taken care of, patients are concerned, one or the other. Without that, you are a procedure looking for a home.

DR. DAVIS: Well, at least in the U.S., there is a process. You apply for a CPT code. And

I have done three of them. There is no reason why CCS can't fit the American group for that one. I mean, the European is different country by country.

DR. STETLER-STEVENS: So would that come under regulatory concerns?

DR. DAVIS: Yes, it is a regulatory --

DR. VOGT: What have you applied for? What is one of the three you applied for?

DR. DAVIS: Reticulocytes, reticulated platelets, and I guess the third one didn't make it.

DR. VOGT: Was that an arduous process or --

DR. DAVIS: No. It's just paperwork. Basically what you do is you say there ought to be a CPT code for this and list the references showing the medical utility. And you submit it to an AMA committee charged with doing these codes.

It takes about a year and a half to go through the process. And if your argument is sound, you basically end up with a CPT code a year and a half later.

DR. VOGT: I think that may be the most single useful thing that can come out of this group would be to get a set of submissions that relate to

quantifying expressions in through flow cytometry onto that master list.

DR. FISCHER: So let me ask you a question.

DR. STETLER-STEVENS: Name one to help with the idea of CPT codes on CD38. I think that --

DR. FISCHER: I have a question. If you get a CPT code and you use things like Lance's CD38 thing or your CD64 thing, do you have to do one for each one of those different ones or can you do one for the quantitative overall or is it going to --

DR. DAVIS: Method-specific because it's justified based on medical utility.

DR. LENKEI: Do you need in the diagnostic, in the same application because I guess clinical trials and separate saturations have some different approaches --

DR. MARTI: Well, in the U.S., I think the vocabulary and language for that in the setting of a clinical trial is whether or not you can get at least cost reimbursement. Usually cost reimbursement isn't talked about until Phase III trial. So I don't quite know how. These would be

seen as *in vitro* diagnostics.

So I would hazard a guess. Now, this is just a professional opinion after some 15 years at the FDA. I can't imagine that we would get cost recovery on *in vitro* diagnostic before it was approved.

DR. VOGT: It's hard.

DR. MARTI: Yes. I'm just guessing. I mean, that is my gut skin take from HIV testing kits to anthrax. I think they actually --

DR. FISCHER: Put Phil McCoy up there on the regulatory. Phil does a wonderful talk on CPT codes.

DR. MARTI: He actually wrote a paper on it.

DR. FISCHER: Yes. He's probably good at it.

DR. LAMB: Well, even if he did CD4, 34, 38 now, you could build a standard CPT code or if you did CD64 now on neutrophil, you could fill the standard, even on phenotyping costs per marker. It's just that you wouldn't recover all of the -- everything you put in for the quantitation.

DR. STETLER-STEVENS: But it would be good to have a CPT code.

DR. LAMB: Oh, I agree. Yes. I'm just saying that is how we have gotten around some of this in the past when we go on and answer on something, is bill for the immunophenotyping, heat and arrest.

DR. DAVIS: Some of this you have to look at cost. My approach to CD64 is that in the U.S. at least, it is standard reimbursement for 88180. The code for each marker is fine to recover the cost or quantitative CD38 or whatever. We have multiple tubes, beads, or whatever.

Reimbursement of $35 may not recover the cost of doing the test. Under those circumstances, you would need a new or different CPT code to get linked to the different reimbursement codes.

DR. VOGT: And we are talking here about more expensive reagents and things like that. So I think that --

DR. HULTIN: And time.

DR. VOGT: And time, expertise.

DR. STETLER-STEVENS: Are there any other areas? One idea I had was for the group was when we get the description back, to go through and try to glean out where we have agreement, write

down where we have agreement, send it out to the crowd, and see if we --

DR. VOGT: We tried that. And it took an hour for them to get --

DR. STETLER-STEVENS: But I think it's very helpful. We send it out. We argue about it by e-mail and fight about it. And certain things we will never agree on. But there are things I think we have consensus on. So let's identify them and let's formalize it, that we reached consensus on X based on what we have gone through. That would be the other --

DR. TAMUL: Call it consensus after review.

DR. MARTI: And basically that will fall to those who are so moved.

DR. DAVIS: And we need to agree that lack of a response is an affirmative.

DR. VOGT: Yes.

DR. STETLER-STEVENS: Lack of response is an affirmative, yes.

DR. VOGT: The default is if you signed up for this, if you don't want to be quoted, you have to tell us what you don't want.

DR. STETLER-STEVENS: Right.

DR. VOGT: You know what, Mary Alice? It occurs to me that could we possibly get a short meeting report into *Cytometry*, maybe before the CCS meeting, so that people would --

DR. SHAPIRO: Are you kidding? You can't get anything into *Cytometry* in less than six months. You can give it a shot.

DR. MARTI: Well, I just know from previous experience that the first thing that happens when you see the transcript and start reading it, you go, "Oh, my God. It's far from finished."

DR. HULTIN: It's a long weekend.

DR. MARTI: It's usually very raw and needs a lot of work because of the way we talk.

DR. VOGT: So we should write this before we see the transcript so --

DR. SCHWARTZ: And then validate it.

DR. MARTI: I mean I am obligated to make some type of summary to CBER, FDA. However, definitely I expect it was somewhere in the mid range. I don't know exactly how it will work. Chuck has been talking about something called perspective in clinical quantitative flow.

Well, perhaps a special issue, maybe

later -- well, let's just see how it goes after we get the transcript, hopefully in ten working days from today, and get it up on our Web site. We probably will take the liberty of just e-mailing it to you. That way you all will have it.

Anything else?

DR. STETLER-STEVENS: Can we think of anything else?

DR. MARTI: Well, you know what? The other day I had promised people that I would bring a list of FDA-approved monoclonal antibodies that are in clinical use. I did make that list, but I forgot to pass it out. It's at the place here if anyone wants it.

I guess on that note, can we officially adjourn this meeting, Mary Alice?

DR. STETLER-STEVENS: Yes.

DR. MUIRHEAD: I think we should thank Mary Alice.

(Applause.)

(Whereupon, at 2:30 p.m., the foregoing matter was adjourned.)