NATIONAL INSTITUTES OF HEALTH

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IDENTIFYING OPTIMAL METHODS FOR

CLINICAL QUANTITATIVE FLOW CYTOMETRY

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THURSDAY,

APRIL 10, 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., Gerry Marti, presiding.

PRESENT:

GERALD MARTI, M.D.	Chairman
RAUL BRAYLAN, M.D.	Member
KENNETH COLE, M.D.	Member
BRUCE DAVIS, M.D.	Member
JEAN-LUC D'HAUTCOURT	Member
RANDY FISCHER, M.D.	Member
ROBERT HOFFMAN, M.D.	Member
ERIC HSI, M.D.	Member
LANCE HULTIN, M.D.	Member
LARRY LAMB, M.D.	Member
RODICA LENKEI, M.D.	Member
PHIL McCOY, M.D.	Member
KATHY MUIRHEAD	Member
ALBERTO ORFAO, M.D.	Member
NORMAN PURVIS, M.D.	Member
JORGE QUINTANA, M.D.	Member
ABRAHAM SCHWARTZ, M.D.	Member
HOWARD SHAPIRO, M.D.	Member
MARY ALICE STETLER-STEVENS	Member
KAREN TAMUL, M.D.	Member
ROBERT VOGT, M.D.	Member
JAMES WOOD, M.D.	Member
YU-ZHONG ZHANG, M.D.	Member

I-N-D-E-X

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

8:10 a.m.

DR. MARTI: On the record.

DR. STETLER-STEVENS: We're going to get them to open this door and to get more space here. We're going to expand out over there. We have the next room and we're going to have that for a break-out room but I think we have more people than we expected and that is good.

I'm going to start out what precipitated this conference. I'm at NIH, and we are doing a tremendous number of antibody-based therapies. I don't know the new protocol that's come on. It didn't use antibodies as part of its therapy in leukemias and lymphomas. You use it in acute leukemias, lymphomas, chronic leukemias. So it's a big focus.

Every six months a new one comes along. They are finding this to be very complex using antibodies alone, antibodies complex to yttrium, the very rapidly degrading radioisotope, complex to ricin. People in England are afraid of ricin. We have ricin at NIH, a pseudomonas toxin. They've--- had some excellent results. I was involved with one protocol using anti-CD22. I looked at CD22 and

CD20 together. I like to look at the two of them. In my hands, normal B-cells are about right here, a nice population. This was a protocol that has looked at the chronic mature B-cell leukemias.

We had three types of cases that came in, hairy cell leukemia. This is where the cells are an expression of CD22 and CD20. CLL, where this is the normal. We had some squanic, marginal zone lymphomas, which are closer to normal. They are treating them with an anti CD22 antibody complex totoxin.

The hairy cell leukemia patients achieve complete response. We detect hairy cell leukemia easily at 0.6 percent of the lymphoid cells which are hairy cell leukemia. You collect enough that they are so abnormal that they pop out. They got to the point where I couldn't detect any leukemic cells. Although we could have acquired a Million events, there was not a distinct populative, excellent response. Their cells normalized. Their immune function came back, excellent response.

CLL had a much poorer response. There was some partial response and the splenic marginal zone was in the middle. It's easy to see that it

relates directly to the level of expression of CD22. They are all refractory hairy cell leukemias. A lot of hairy cell variance. CLL patients again are post treatment, all of them are post treatment because this is not a first line treatment. This is an experimental protocol. It was obvious to me that it corresponds to the level of CD22 expression.

Looking at various leukemias and lymphomas there is a spectrum of expression. I think that the level is going to be important in determining responses. The same person that's using CD25 complex of pseudomonas toxin and using it again in CLL when there is dim expression in various leukemias and lymphomas. In adult T-cell leukemia and lymphoma especially with HTLB1, high level of CD25 expression and it responds very well to CD25 therapy of any kind, complex to radioactivity, toxins, whatever. So there is an obvious correlation to the level of the expression of the antigen.

At that point, I decided we should start doing some quantitative studies in these patients. We then went on to some more complex ones like Hu1D10 which is an antibody being used in

therapy. It's not as obvious a difference, but there are differences and within the same categories. In CLL, some patients have high levels, some are negative and some patients are low and intermediate. It's a big spectrum. So we started to look at expression of this antigen. We are trying to do some quantitative flow.

It became immediately clear. I'm going to show you all CLL patients. This is all the same disease. This was just in one week's worth of some cases we picked out where we had some funny results. I showed this to Gerry. This was because medical technologists said I can make those values change by how I look at the data. It's already acquired. We were looking at the geometric mean. This is looking at a lymphoid gate in a patient with CLL. He's post treatment so it's not an overwhelming CLL. He's got about 56 percent T-cells. When we look at Hu1D10 which is this variably expressed one in the non T-cells, you get a geometric mean of 349.

DR. SHAPIRO: What is the CD?

DR. STETLER-STEVENS: I'm sorry. This is CD3 versus Hu1D10. This is CD19 versus Hu1D10. The same two. We have 3, 19 and CD14 in with

Hu1D10 so that if you look at it, it's expression. Interestingly with this antigen there seem to be toxic reactions in some patients and it has to do with expression of Hu1D10 on other immune cells. T-cells and monocytes might be important in this, they also get stimulated by this antibody. So we are looking at this. But I can find that if I look at the non T-cells I get about 350 as an X geometric mean. If I look at the CD19 positive B-cells, I get 502 and it's in the same two.

DR. SHAPIRO: Say that again please.

DR. STETLER-STEVENS: I'm looking at here CD3 versus Hu1D10, same two. So by looking at it by this way in a lymphoid gate, I drew a scatter gate RA based on the lymphoid cells and I'm looking here. This is CD3. These are the T-cells. These are the non T-cells, lymphoid cells. Basically it should be the patient's tumor. I also run kappa lambda tubes of CD19 so I do look at this another way. I'm going to show you how that leads to problems. So for the non T-cells, I get 350. Someone might use that as a method of looking at it. For the CD19 positive cell same tube, I get a geometric mean of 502, but in the same two.

DR. VOGT: So that's not an

inconsistency. That's two different populations.

DR. STETLER-STEVENS: Not necessarily. It is two different populations. There are some differences in there, yes. There could be some NK cells in there expressing. There are various things. Then when I do another 19 specific gate and I look at Hu1D10 and the 19 positive cells, I get with a gate, not all the lymphoid cells. This is gating strategy making my geometric needs. I get 759. Whereas when I was looking at all lymphoid cells, CD19 positive, Hu1D10 I got 502.

DR. MARTI: So no matter how you slice that regardless of whose calibration curve you use in what lab, what instrument, what country, a 200 channel difference in the calibration curve has to give a different antibody finding.

DR. SHAPIRO: Wait a minute. The fundamental problem here is that you have to do the defined population. That is with different gates.

DR. STETLER-STEVENS: Yes, but this is one thing we need to talk about. How do you approach this.

DR. MARTI: Which gate do you want to use?

DR. SHAPIRO: But it seems to me that

if you are trying to define and theory and you are looking at some population and you are trying to relate a quantitative antigen expression to tumor response, then however you define it the first thing you have to do before you attempt to do any quantitation is to develop robust criteria to define the population you work with.

DR. BRAYLAN: That's right. What we did with CD34. You have to define a very strict strategy.

DR. STETLER-STEVENS: We've gone with using a template that we do not stray from in CLL. If you are going to compare between patients it has to be exactly the same way, analyzed exactly the same to make it comparable.

*DR. LENKEI: Yes, but I also think specifically in case of some audiences --

DR. STETLER-STEVENS: We have a lot to talk about. Now I have another patient. They are going to be the same gating strategies. This is CLL. As I said I want a tube with CD5, CD19 and kappa in one tube and lambda in another tube, the only thing different between the tubes. Nineteen, Hu1D10 and CD5 and then one tube kappa, the next tube lambda. It's the same fluorochrome kappa in

one is FITC and the other one lambda is FITC. Same patient. Same cells with a pipette repeater put out. Everything is the same except one has kappa FITC and the other one has lambda FITC.

When we look at Hu1D10 here there's a lymphoid gate, exact same gate. This actually agrees pretty good. The non T-cells I have about 300 and the CD19 positive cells I have 333. This is with kappa.

Then I look at lambda and it's changed completely. If you look at this versus this, you don't need to look at the numbers. This is a standard setup. The same blood, the same antibodies except one has kappa and one has lambda. There's a big difference. And it's reflected. Look at this. I don't have to tell you that there's a big difference in the geometric. This is with kappa and this is the same patient, same antibodies, with lambda. It's the same patient, the same antibodies. Look at that.

DR. D'HAUTCOURT: Which is the light change of the tumor cells?

DR. STETLER-STEVENS: I have that. Kappa.

DR. FISCHER: It's not the different

antibody exposure. These are the same two antibodies with two different tubes. These are infected with a third antibody.

DR. STETLER-STEVENS: This is kappa. The tumor does express kappa.

DR. LENKEI: ** the compensation.**

DR. STETLER-STEVENS: Do you think we have a compensation issue?

DR. LENKEI: Yes.

DR. STETLER-STEVENS: There's the same fluorochrome, etc. Or it could also be interaction between antibodies because the tumor is positive, for one, of the light change. The tumor being positive makes a difference. It's going to shift your values.

DR. LENKEI: I never thought it mostly compensation.

DR. TAMUL: Are the kappa lambda antibodies monomodal or bimodal?

DR. STETLER-STEVENS: They are polymodal in this tube.

DR. BRAYLAN: So your percentage issues are ready to discuss.

DR. STETLER-STEVENS: Yes.

DR. BRAYLAN: Let's not discuss the

specifics.

DR. STETLER-STEVENS: This becomes a test. People are going to do this in a lot of laboratories. I believe antibody-based therapy is going to be with us for a long time. It makes sense. What are they going to do when they have a bimodal situation? And this gets to reporting. It goes from all the technical issues of compensation, antibody combinations, gating strategies and reporting.

Having shown this just gleaned together quickly in one week the complex things we saw, I showed to Gerry and Gerry said we have to do something about this. I really believe that this is going to help me stay in flow until I retire. I see this as something that we're going to be able to do and make money doing. I want us to do it right and not have people end up saying well it showed absolutely no correlation with prognosis. Why? Because they just did what I did. They cranked out stuff and it doesn't correlate.

DR. BRAYLAN: Is there a great demand for a quantitative assay for these things or is it just that something semi-complicated will do?

DR. STETLER-STEVENS: For the Hu1D10

they want to know because they want to know the levels of all the cells. They are research people. The one person in the Hu1D10, Wyndham Wilson, is astute and he's aware of these issues. The CD22, I had to explain to this guy about three times what we can do with quantitative flow. He said you mean I can stop doing the radioimmunoassays to determine levels of CD22 expression. He said oh, that's really good. But it takes Millions of cells.

DR. VOGT: That's okay. Actually being able to correlate an RIA result will be a very valuable thing.

DR. STETLER-STEVENS: We're going to try to go and compare our data. He would like to move to us just taking one blood sample that are practically exsanguinating a person to get a large enough sample to run more radioimmunoassays.

DR. SHAPIRO: The fewer the cells the less disease they have remaining. It's like if you try and do blood cultures and you get two units blood.

DR. BRAYLAN: Some people would argue that we really don't have very strong evidence that responds to therapy relates directly to antibody binding sites or expression of the cells. Going

back to the example you gave, they will argue the response doesn't necessarily relate to the expression but it relates to the number of cells in the simulation. CLL having the highest marginal and hairy even less so there is no multi-variate analysis for that. That's the agony. On the other hand, you can't prove something that you cannot measure.

DR. STETLER-STEVENS: We get patients that come in actually with high numbers with hairy cell leukemias because these are patients who have failed therapy off the hairy cell variant. I have seen a lot of hairy cell variant. At NIH you see a lot of weird things. I don't see a lot of common things. Every time we get something common, we're overjoyed. So they come in with high numbers. I would say that it's not it, but we don't know until we look. If we look the wrong way, it's going to be like S-phase. People say S-phase is totally worthless. And if you do it bad, it's probably worthless.

DR. MARTI: Mary Alice, my comment to that is that if we use the expression of CD20 on CLL and based on the experience that some patients come into the clinic and they say in Miami they

said that my cells didn't express CD20 and therefore the oncologist said I couldn't have Rituxin. But I went to Conti Ries lab in New York and they said that 60 percent of my cells were positive and he would give me Rituxin.

Now my answer to that is don't even look at the CD20. Give the Rituxin and see if it works. That's the clinical approach. The problem with that says is that there is a lot of interrogatory variation.

The second thing I want to make a comment about is Hu1D10, the class two antibody. Mary Alice alluded to it. This appears to be a very effective antibody in indolent lymphomas. Unfortunately in the first 20 patients that were treated, there were three episodes of hemolytic uremic syndrome. That protocol was put on hold and those issues are being worked out.

Those 17 patients that didn't have the hemolytic uremic syndrome, they are beating on the door wanting their next course because it was so effective. Part of the reason for wanting to quantitate is and, not so much on whether or not the tumor cell is positive, but what about the other cells. It looks like the endothelial cells

and monocytes could be playing a role in precipitating this HUS.

DR. STETLER-STEVENS: There are differences in the patients who had hemolytic uremic syndrome. They had higher level expression on the non-tumor cells. Another thing in the Ricin toxin therapy they found that patients who had no circulating tumor cells had a lot of toxicity to Ricin. There may be an importance into having too much toxin floating around.

Whereas if you had high affinity and it's quickly taken up, then you won't have too much toxin around. So the dose level may need to be adjusted according to the affinity number of molecules even available in the blood.

DR. WOOD: What I was going to say is that there are a lot of questions that are involved here. The biology is a complex issue. Unfortunately there are a number of serious obfuscations that are going on at the very fundamental level in just detecting light from the instrument. That is how people do these multi-color experiments without batting an eye. The whole issue of the fundamental theory behind the compensation is what does it mean to compensate.

That is are you compensating the dyes? Are you compensating dyes on the probes or the actual antibodies themselves? Are the two antibodies, if you are looking at multiple antibodies, are they really truly independent when you are doing the compensation?

Also just something as fundamental as the log display is an enormous confusion factor. In fact, I dare say that the flow community has truly looked at it careful enough to understand how sometimes they are being mislead totally by the presentation and the display. And so, there is at the very basic level a lot of problems, a lot of confusion that's occurring that is totally confounding the biology.

Without going back to the first principles of looking at the light going in to the flow cytometer, the light being converted to the electric signal, there's a lot of electronics that is going on that people just look at as black box and not think about it. Then all of a sudden, magic symbols or magic boxes appear on the screens. And there are a lot of assumptions being made that unfortunately a lot of them are very misleading. That just makes the biology an impossible issue.

DR. MUIRHEAD: I'd just like to second something that Raul just threw up at the end which is you can't ask the question of in which cases is there a real difference in the biology that has implications for treatment strategies or patient management or anything else unless you can get the factors that Jim and everybody else is talking about under control.

In some cases you may only need a semi-quantitative readout. In other cases, the quantitative readout may be really important. But you can't even ask the question unless you can compare data in some common scale across different laboratories.

DR. LENKEI: I should say that today not the instruments are the main concern is really talked about for some reason. They end up mainly to set up instruments. For examples, I refer to the ones with experience. They are so good that when they come and I check with some of my beads and theirs and they are exactly the same. I don't know, I don't even know if the stain is the same. The problem is still that protocols, how we stain our cells, should the compensation when you have an activation. (Inaudible) This is my main concern.

DR. TAMUL: The problem is the different patient cells will behave differently even with the same antibody.

DR. SHAPIRO: Again another point that's come up here is that compensation is the enemy of quantitation. But as Jim McGall can tell you that the cross talk -- what you're trying to compensate out of the signals -- becomes noise on the signal that you have left. If you are trying to do accurate quantitative flow cytometry you should probably have a channel while you are measuring it.

The other thing that we got from the DNA analysis in breast cancer and other things is that if it's not going well, it's not worth doing. This is a complex problem. I think it's fair to say that the instrumentation in the reagent development has progressed to the point where we have a pretty good shot at doing these measurements on the fanciest machines that are out there. What we should aim for is figuring out how to do this well and then figure out how we can do it with the machines that are out there right now.

DR. LAMB: I'm trying to boil the last 15 minutes that I've here down into something

practical. I'm thinking that Raul is right about the practicality of it but if we get into a world that is defined more with tumor associated antigens and that sort of thing where quantitative flow is going to be even more important to be able to decide whether and if and how much therapy.

It's already started of course with the Rituxin and Bexar and the anti-CD 22 agents. I think that the methods are going to have to be developed centrally. This stuff is going to be done in a few laboratories in the country like Schiller Psychology Group has two reference laboratories that decided to go on protocol. Then everybody else will catch up.

But I think it's probably this group of folks who are trying to put together methods that will work across instruments that will develop the procedure. That procedure will be done in tightly controlled situations and tightly controlled laboratories before it goes off. So you have to crawl before you can walk.

I don't think we can start thinking about exporting this technology to every pathology lab in the country, although the people who make the beast would want to see that happen. At this

point, you just have to concentrate on getting the method right and in place in five or six laboratories that can be reference facilities.

DR. FISCHER: It has to be important in the end of make it exportable because I know that at least with our trials at NIH that we also have sites around the country that are part of the overall trial. If they're not doing it the same way we're doing it, we may have the best technology and the best system in the world, but if they are not doing it the same we are we can't compare our results.

DR. STETLER-STEVENS: I think it's not going to be done in a little lab in West Virginia.

DR. PURVIS: I think it's important to go ahead and note at this time though that there's a number of pharmaceutical companies that are now recognizing the utility of doing the quantitation. We have a number of clinical trials that are currently going on with a number of pharma companies where quantitation is of the antibody binding saturation determining dose levels. Doing a BK/BB off of a flow cytometer is one of their primary measurements. Those protocols are being developed and utilized by the pharmaceutical

company and as those monoclonals hit the market that is going to be one of the measures that they are going to want.

DR. VOGT: That's the question. I was just going to ask you to comment on that because it's always struck me and Gerry and I have talked about this. I didn't say that because Gerry is not allowed to talk about some things. But I wonder how much data there is in the pharmaceutical industry relevant to this that we don't know about. At least with Rituxin data, it did not look like when it was rolled out that there was an attention paid to this. Is that same mistake going to be made as these others come out?

DR. PURVIS: There are a number of monoclonals that hit the market that quantitation is not done at this level. There is no corollary to should we give it in this disease case or this expression level.

But the new monoclonals, the campass, there's a number of them that we're working on that I know that there is development of corollaries. And those will be in relation that they will provide help to the clinicians. That will be one of the marks that they'll be asking the labs to be able to provide

some information back on. You have to establish the protocols now that everybody can agree to. Because here we are. We're doing it in our lab by our protocol and get very good results. If you do it by a different protocol when it hits your lab, it's going to be useless information.

DR. VOGT: Then, Norm, that leads to will the drug company allow you to share your protocol. Is it going to come with a package insert on the drug because there is no sense in reinventing the wheel and certainly no sense in reinventing the flat tire.

DR. PURVIS: I think that's what we're going to discuss today.

DR. VOGT: How about establishing a guideline of this is how we're going to approach quantitation in the future. I'm not going to say that our way is the best way and the only way. It's not. There are a number of different approaches that we can take for quantitation. What we have to do is come up with some kind of guideline so that we can get good results and avoid the mistakes that we're making with DNA that now we're having to go back and trying to correct for. Bad data is going to kill this.

DR. STETLER-STEVENS: This segues into the goals of this meeting which are to identify the problems and identify possible solutions. We're going to meet again in November at the close of the Clinical Cytometry Society Meeting. They are going to sponsor us insofar as they will provide the rooms for us.

Hopefully, in the time between now and then, we will have identified studies that need to be done to look at things across laboratories, perform some studies and come to our final conclusions by next November. That's being optimistic. Or at least have a basic guideline that we can work on.

DR. SHAPIRO: Do you have a statistician report?

DR. STETLER-STEVENS: Anybody?

DR. SHAPIRO: The reason I ask is that if we look back and there's a fairly long experience with both flow cytometry and even quantitative flow cytometry in the AIDS clinical trials group. What we know from that is that it has been verified by physical analysis, is that labs that are doing badly on QC, you can take the data and their information does not have the same

prognostic value as data from labs that are doing well. That's one point that we picked up.

The other thing that we picked up from the CD38 studies is that as hard as it was to get labs to agree on simple CD4 counts is that it's that much harder to get them to agree on quantitative analysis. In that framework, we know that we have to start with a few labs and figure out how to do it right and then start spreading it out to good labs. You may never get to the poor labs. That's the way it works.

But from the ground up, the problem is there is this exchange on the cytometry abilities a few months back. How can I implement this vertical for detecting renal residual disease in myeloma. And, in doing that in one haul in a lab is never a good idea. You make a decision with implementation care. You need protocols and you have to figure that out. The bio statisticians and clinical trial statisticians are equal important components of the design of this protocol. So if you are going to play this game, we should get somebody on board for that sooner rather than later.

DR. MARTI: That's a good point, Howard. I certainly will make an effort at our

institution both at the MBA and certainly within the Family Study Section at the NCI that they are top heavy with biostatisticians.

DR. SHAPIRO: I used to be a biostatistician at the NCI.

DR. VOGT: In fact if there are clinical trials going on, there are already biostatisticians. The question is have they been engaged in looking at this.

DR. SHAPIRO: We might want to see if we can have some liaison that can go and see the people who are doing the statistics for the NCI. They are up to speed on what that is about.

DR. MARTI: Just an aside comment about the pharmaceutical business, one thing that I can certainly speak about, and this is public information, was one of the things that held up in getting Herceptin antibody to market was the very issue of quantitation. We're not talking about flow. We're talking about one plus the four plus on a histopathological segment color assay. Believe me there was some arm-twisting and torturous discussion about the so-called fourth phase of drug development and that is a post marketing agreement. That's the way that one got

out.

For better or worse, I think that the FDA will, in its infinite wisdom, will continue to look even more harshly or strongly at these kind of quantitative issues. I actually think that this problem has emerged. I would call it a problem of quantitative flow in setting of four colors or more. I actually think for those who live and die on the word quantitative flow that this is a gift to figure out this set of problems.

What Jim was saying earlier about the basics -- perhaps in my case I forget the basics. So I have to keep returning to them because the basics just get more complex. One of the examples is that at least with the CyAn and with LSRII, and probably the aria-- I don't know about the LSRII -- but anything that's using digital signal processing guess what? Negatives now become a very important issue.

DR. SHAPIRO: We got that fixed.

DR. MARTI: Certainly enlighten us because now it becomes an issue. Not an issue, it becomes a reality, a possibility. That's just one of the things that jumped out at me recently doing something as trivial as if you thought titrating a

reagent so-called QC of the reagents. At a research lab, many of these reagents are made. They are not commercially available. They know QC in a much different way than a microlab does.

DR. FISCHER: It basically doesn't work or not as the QC in essence.

DR. MARTI: For commercially available reagents, it's not a question of whether it works or not, although sometimes it is. It's often a question of overstaining is a very dangerous thing in multi-color worlds. Just think of all the H&E sections or blood films that you've look at that are overstained. How do we ever see anything if something is overstained? It seems to be the same problem with fluorescent dye. It really is relearning the old things in spades over again. They become really important.

One of the things I hope that we walk away from this is compensation controls. I might even be so bold as to say compensation standard. In this illustrious group, we ought to be able to come up with a suggestion of a standard. I know that's a dirty word. Controls are preferred. I just throw that out for thought.

DR. STETLER-STEVENS: Beyond this

scope, we have a big problem that we need to work on, kind of clinically. If you have seen the agenda, do you have any additional topics to add? Actually, you can at any point jump in. We're going to talk about everything from sample preparation to --

DR. VOGT: Why don't you run through very quickly the agenda topics?

DR. STETLER-STEVENS: All right. This afternoon, we're going to talk about sample preparation, staining, the analysis. We're going to talk about QC tomorrow for instruments, reagents, sample prep, standardizing data analysis, reporting of results, identifying reporting samples, specific anomalies.

At any point you can jump in with something as it occurs to you. While we need to talk about this, we will probably break into working groups because I thought it would be the techies and the pathologists mainly because there are some things in a techie group I don't want to hear about until it's already decided. Just tell me what works best. With that, why don't we move on to the -- proposed guidelines.

DR. HSI: Is there a way in the context

of what we're going to doing today to think of a specific application? If you start generalizing too much, it will be very difficult to do anything. The reality is the things that we are all talking about are rare. Leukemias in a setting when you look at cyto tumors are rare. We may focus on one little thing of which the actual implication is very small versus something that's more generalized. Think of an application that's more full run of flow rather than thinking about the different specific clinical trials.

DR. STETLER-STEVENS: Leukemias, lymphomas, psoriasis.

DR. HOFFMAN: Maybe sepsis is something.

DR. STETLER-STEVENS: Sepsis.

DR. LENKEI: I see quite a bit of this based on one of the antibodies. I see a doctor's continuous development. I have been engaged in projects from these companies for three or four years now. It's a field that's appeared. I can tell you that the pharmaceutical company engaged in my laboratory found another laboratory in Europe really known. At the end of the one year, I was wanting to their papers. They called and asked me

to help the other laboratory because it was such that the notes couldn't be used. It's a very serious issue because the patients were called for receptor separation. If you don't have good protocols and standardized methods, they were using the same reagents, but their protocols were different. Then the results after one year couldn't be used by the company. They are very aware what it means.

DR. STETLER-STEVENS: It's not just antibody-based therapy. There are other immune function studies and sepsis groups are going to talk about these studies. There are other applications. My focus is cancer because I'm in the Cancer Institute but there are other applications. Bruce.

DR. DAVIS: Before we get too far, I think that it's important also to agree with what we are trying to accomplish after two days. If I understand the conversations and statements, don't we want to establish principles of quantitative flow, a way to verify the principles. That's what we're going to do between now and November and then establish who is going to do it and how. So is somebody writing? What I'm thinking if we don't

have a protocol by the end of tomorrow we would have just gotten together a couple of days of brainstorming.

DR. VOGT: We've asked Howard to be the summarizer and raconteur. But that's a very important point, Bruce. Maybe we should just ask Howard instead to put it up there.

DR. MARTI: I would also make a comment that we are going to make a transcription of this meeting. There are microphones placed in the rooms strategically.

DR. VOGT: By Homeland Security.

DR. MARTI: The transcriptionist has asked two things. One it would be useful, and I haven't done it at all this morning, is to identify yourself as you speak. The second thing is if you can speak from the diaphragm and not this guttural stuff that we normally use. Technically speaking we are obligated by contract to have this available in a public website in ten working days after the meeting. You'll get an opportunity to edit it however you like because I will not edit it before it goes out. I don't think so.

DR. STETLER-STEVENS: But we can certainly identify problems and things that we want

to address.

DR. VOGT: Mary Alice, don't you think we could actually start writing some things down up there on one of those two things. That might not be a bad use for a flip chart. I did some of these other applications. Autoimmune disease is a public health problem as opposed to leukemia perhaps.

DR. MUIRHEAD: But I don't think the problem is lack of application. I agree very much with the comment that if you are going to try to put together working groups to generate data to say if we try this as a common protocol for this application, do we get more consistent results among laboratories. I think you're going to shoot yourself in the foot by trying to make a given protocol apply to every kind of application because the critical issues are going to be different for at least some applications than others.

I would second the comment there that we can think about some of the common issues that are going to be found no matter what kind of quantitation you are doing and what kind of reagents. When you come down to putting together the working group plans for what kinds of protocols are going to get saved, I think you should focus

them around, I know, pick it, two or three specific applications. Otherwise, I think you do run the risk of being so general that you can't solve the problem in general. It has to be solved in a context of a particular application.

DR. HSI: I think it also reads to other non-flow people what's the utility and why are you doing this. So, if you have some more broad ranging applications than just a single disease, you know, with leukemia, that it makes it more relevant.

DR. SHAPIRO: There's a problem here which I alluded to many times. And that is that, fundamentally, we're all used to the content of offering drugs. What we are dealing here with is talking about diagnostics. If you just look at the economics of clinical flow, automated cytology automation, flow cytometry and hemacytometry really got their start on pap smears of which there were a 100 Million done every year and differential counts of which there are 50 or 100 Million by the end of the year.

If you look at the most common fluorescence flow cytometry diagnostics, and you see the workout, there are probably no more than

1,080,000, probably not 2,000,000 done a year. After you get down to leukemias where you have 10,000 cases a year, this is literally a morpho-diagnostic. If you focus too hard on specific application, somebody is going to decide that they are wasting money.

While it's true that you will have to pay attention to different aspects of the problem as you try to work out the quantitative protocol for one clinical application or another, we're still at the point where we have to define the basics that can go into the formulation of the protocol to be doing with any benefit. We have to say while any particular disease we might be working on is a morpho-diagnostic and when you add them all up, you are dealing with problems that affect large numbers of patients and you could have a substantial effect overall on healthcare, if you get it right.

DR. FISCHER: Doesn't it also come down to the fact that possibly because we haven't done this kind of thing successfully in the past, and I know years ago, Gerry, you organized another one of these meetings, to my knowledge, nothing concrete came out of that. We all got some new ideas.

Until that happens, the whole technology is going to be underutilized. There is probably more instances where it could be utilized than it is. To say that only one Million or two Million of these things are done maybe it's because the technology hasn't been recognized because we don't have the kinds of standards that we're talking about.

DR. VOGT: I think that's true. I'll take this opportunity to start my talk now. Part of the problem here is me. We actually did have a very good output from that meeting that Gerry had in that series of meetings that occurred. They were compiled into the special issue of cytometry. More importantly there were two very specific things that came out of that meeting that have just now reached fruition.

One is the NIST program for standard reference materials for this kind of analysis. That is a concrete block that we needed to have in place for some time. We have three folks from NIST here, Dolph Gaigolos who was the leader of this effort overall and his colleague in the lab, Colleen Lange, and Ken Davis. That's one of the concrete things. That came directly out of Gerry's

meeting. Gerry met Dolph at that meeting is my recollection and talked to him about that.

The second thing that came out that is specific is the NCCLS subcommittee. That's been held up by me for more than a year. It should have been out a year ago but it's about to come out. It is very important that when we have these meetings and say we're going to do something that we get it out there. I think that point is extremely well made. But those things are going to be out. By the end of this year people will know about them.

Just to add one more little thing. There are trials going on right now in Type I Diabetes using humanized CD3 antibody to try to prevent progression of new onset disease. Two to three in a thousand children born in the United States will get Type I Diabetes. It is probably a preventable disease. We know the risk factors now that will allow us to hone in to a 50 percent predictive value if we did general population survey. If something like that hits, it will change everything in terms of the marketplace for this kind of stuff. I think it will hit. I think Type I Diabetes will become a disease like smallpox used to be.

We need to be very alert to the opportunity and we need to be ready when we strike. I agree with Kathy absolutely that it's still going to be case by case no matter how prepared you think you are. When you get down to that individual case, you're going to find some things that are particular to that case.

DR. MARTI: I would also add to your comment about the outcome of that meeting. In my experience at the FDA, industry refers to that special issue more frequently than we are aware of in the scientific community. The other thing that came out of that meeting which was not really addressed was the total area of positive cell control. An example would be stabilized whole blood. We haven't really specifically addressed that, but at that meeting that was very much encouraged.

I would also point out, just for the record, that prior to that meeting we really only had one or two standards or controls. All of you are old enough to remember finus nuclei. We now have eight or ten products on the market that can be used in this area of standards and controls.

DR. LENKEI: I would also like to say

that I agree completely with Howard because after we go to specific applications, it's amazing. When I say basic applications I don't think so much about calibrated. I am talking about other procedures. I agree with them. I agree completely. Now nothing will tell you with specific applications if the basics are wrong.

DR. LAMB: Absolutely. Mary Alice, the other thing is this is a big animal that you are trying to get your arms around here. You can take something like CD34 which we've had 40 zillion consensus conferences on around the world, and I still, after just completing a study with four major core blood centers looking at very methods of measuring CD34 and then taking two manufacturers methods of keeping my hands off of them and just letting them run the way they are supposed to run without tweaking the dot plots, it's still out there. You have published data on quantitative flow cytometry measuring the same thing on the same cell that differs by law.

What it boils down to is the NCCLS and Bob and his Argus group there have to come up with a procedure to where I will trust both the result from the laboratory run by the person sitting next

to me and they will trust mine implicitly because we use that technique. We don't get in meetings and say that manufacturer's beads are crap or that manufacturer's beads are no good or we can't use this. We have to do this ourselves because we can't sit at the reference lab because doctors want from us the data. Even above applications and everything else, the first thing that has to happen is you have to be able to do the basic things, to calibrate the instrument and stain the cells correctly which is something that people still aren't doing.

DR. STETLER-STEVENS: We're going to need input from manufacturers of various products to support this. If it's just throwing a dye in, we're going to need everything even up to including software to help people to approach these problems. It's going to be a market for the future. We're going to need to know what we're doing and we're going to also have the right products to be able to do it.

DR. VOGT: Let me run through this. There are a number of people that are participants as members or advisors or observers on this committee. I will be speaking to the choir to some

extent. They probably wonder what's happened to the hymn book.

This is my term "quantitative fluorescence calibration." You can also call it "quantitative flow cytometry." You can use the same acronym. Basically we're trying to get toward the direction that Jim was talking about and that is really what are we measuring and how are we measuring it and how do the standards and materials that we're looking at "measure and the word now, how do they relate to the process.

These are some selected slides from something that I put together and gave to Howard that he then improved on and presented last week in Belgium at an international meeting that was hosted by a European group and that our colleagues at NIST had been involved with. The basic idea is trying to get these arbitrary scales or relevant fluorescent intensity scales into some kind of reproducible stoichiometric scale.

Stoichiometry, I actually looked it up as I was writing, basically says that if you start with this set of reactants you wind up with this set of products. All the atoms and electrons that were here have to be over here somewhere. It is

the balancing of those reactivities.

As it applies to what we are doing is what I call ligand-binding assays. The NCCLS parent committee that our so committee is out of is the Immunology and Ligand-Finding Assay Committee. Basically what we are talking about here are binding assays. The ligands in our case are the fluorochrome conjugates if you are talking about typing.

As I mentioned the big leg up since 1997 is that NIST scientists have gotten involved in a very big way. The first standard reference material in 1932 which is a fluorescein solution very clearly characterized after being synthesized by Duzon and his colleagues at Molecular Probes is now for notice available March 2003. It was on the NIST website three or four weeks ago with an expiration date and no price. Then a week later the price appeared so now you know that it's real. What is it, Dolph, $100 or $200 per kit?

So one of Dolph's fears is that these things will sit and gather dust. Nobody cares about the money that this brings into the Federal Government but they do care about the indication of interest from the community. I would like to

encourage everyone to buy a kit. I'm serious. You think I'm kidding. I'm dead serious. Buy yourself a collector's item because it is my impression that NIST does not intend to do this again.

An SRM is a big effort for them. Future things will be more along the lines of traceable materials. Dolph, is that correct? Where they will use NIST procedures and available NIST standards to make things traceable. That reference solution is now available.

Now the idea of the quantitative fluorescence is that we would use these solutions as the calibrator for our particle measurements. So the companion reference material that we need to go with this is the fluorescein labeled microbead.

By the way, I have this PowerPoint presentation. It was on the computer. I have these little chip things, flash drives, so if people who are giving presentations that have PowerPoint, if you have your laptops with you and the presentation is on it, I'll give you one of these and you can copy it on to it. Conversely I can copy everything that I have and everything that's presented here on to one of these and then you can copy it on to your machine. So we can have

a little virtual network.

DR. FISCHER: Can that be made available to the rest of us who aren't giving presentations?

DR. VOGT: Absolutely. That's the intention. We can this posted too. We will certainly make that available. So the companion material is a reference material which is a little easier to get through the system than standard reference material. It's 1933. Dolph and his colleagues are working on that right now.

They are beads that have been custom synthesized by Bangs laboratories and are surface labeled with fluorescein so they have the requisite environmental responsiveness. Dolph and -- now later are working on the exact methods and translation of how you get from the solution to the particle assignment. Dolph, is it fair to say that within in the next year that those beads you think will be available?

DR. GAIGOLOS: That's what we intended to do.

DR. VOGT: I can say that in my years working with the wonderful colleagues that I've had the chance to work with, Dolph has just been

extraordinary in his thoroughness and ability to get things through. He never thought too much about the difficulty of all this until he hit the beads most recently now. But we're getting the best possible effort at NIST here. That's a big lift up.

DR. DAVIS: Bob, are you saying that the procedure is still under development?

DR. VOGT: The exact assignments? The exact procedure for assigning?

DR. DAVIS: Yes.

DR. VOGT: Actually thank you for asking that, Bruce. There are a series of papers that are in public domain from the NIST website. I have hard copies here. I have the PDF files with me. Again I can give them to you and they are downloadable from the NIST website. There are three papers, one of which will be, at least one, maybe two, will be an the appendix in the NCCLS document and they describe the procedures both theoretical background, the computations, mathematics of all that and the practical methods. The third paper is called "Practical Methods." Those methods have been published.

Now the exact micro-implementation of

those practical methods is again going to be case by case. It's my understanding that's what Dolph and company are working on now. I'll give you an example in just a second -- you know, the devil is in the details. Again case by case you will need to look at that. But the general methods now are in print.

DR. LENKEI: I think you hit one of these on time, to look at the study of the products on the Internet page -- because the terror is so - and you have to look out for the dangers. I had a lot of catastrophes in my other laboratory. Before you assign and you know exactly that this product can be used, you have to have one year to look at the study, and the Internet study.

DR. VOGT: My comment on that would be you are certainly correct and I go back again to what I said that we have to trust each other and that's so important in science. I'm quite serious about that. I do remember a guy named Gorbachev and I think his motto was trust but verify. I don't think we will ever get away from the need for the second part of that.

One of the things in the NCCLS guideline is we do talk about the need for

expiration dates, stability and so on but we also say caveat emptor and that kind of stuff. The person who is using the material needs in their own laboratory to also know that it is stable from day to day.

The basis of the assignment of these values, Howard worked this up and then I went back and changed the green to orange since this was mostly Phycoerythrin. If we excite a solution in a cuvette and get fluorescence, we'll have instrument factors that go into the illumination, the energy providing the excitation. Then we have fluorochrome factors and the saline factors or the concentration of fluorochrome. The absorptivity expressed as an extinction coefficient and then the quantum yield.

Then in addition, and this was the big break through to those of us who are not like Jim and Bob Hoffman that are not optical physicists, we always worried about the fact when we have these differing emission spectrum fluorescence which as you all know are broad and tend to vary with respect to environmental conditions and so on. How do we ever assign standards -- whose bandwidth are we going to use? What is the standard? At one

point maybe we could come up with that there is going to be a master bandwidth filter that everyone can use.

When this got in the hands of people that knew what they were doing, they said no, you just integrate over the entire emission spectrum. In the assignment of the standards that can be done because you have time to integrate. Now when particles are going through laser beam, you don't have time for that. There's a difference here between this kind of measurement and the analysis on the flow cytometer.

What Dolph is working on - please, Dolph, correct anything I misstate -- the assignment of the standards will not depend on spectral matching. They will depend on the integrated emission energy across the entire emission spectrum.

One of those devils of the detail that I was going to mention. Gerry and I a couple of weeks ago were talking about this and Gerry said how do they know when the emission spectrum begins and ends, anyhow. I thought about that and said I don't know about that either. I asked Dolph. He groaned for a minute.

They look at their noise measurements in the background and they have a mathematical procedure. They actually take their raw data and model the emission spectrum based on this interaction with the noise levels and everything so that they get a defensible amount of energy that is something that is optical physicists like. These are the kinds of things that are going on behind the scenes.

Then, when we go to read the fluorescence, there are a whole lot of instrument factors. They appear in the measurement equations which are developed in this series of papers. In using state-of-the-art detector, we wind up with a relative fluorescence intensity value from this cuvette solution measurement.

We can do the same thing on a suspension of beads. If the devil is in the detail, then this is hell because there is just a tremendous amount of detail in here. But Dolph and colleagues have been hard at work on this and they are tackling the problems one by one to make sure that these assignments can be scientifically authentic.

If we saw that we got the same

fluorescence reading in a relative sense whatever our scale is between a one picomolar fluorochrome solution which contains about 600 Million molecules per ML and a fluorochrome labeled bead suspension that contains about 600,000 beads per ML and those two give us the same reading, then we have equivalent fluorescence. Since the fluorescence of 600,000 beads per ML is equivalent to the fluorescence of 600 Million molecules per ML, each bead has a value of 1,000 molecules of equivalent soluble fluorochrome.

Now this is nothing new to most of you all in concept. This is what Abe started doing back in the 1980s. I don't want to say now it's being done right but now it's being very carefully with attention to all the details and so on. What we will find is the values that Abe used for fluorescein 20 years ago are just about the same as the values that come out of this SRM. Fluorescein was always pretty reliable as a standard.

Now from the standpoint of trying to getting antibody-binding capacities, we need to relate this to our fluorochrome ligand conjugate. I've just come up with this FLC thing, because I just like saying it. Once again the whole thing is

based on equivalent fluorescence.

So if we have a one picomolar fluorochrome solution and two picomolar fluorochrome ligand conjugate solution, that is to say the molarity of the conjugate molecules is two picomolar -- I bet there are a bunch of devilish details in that -- then, if they give us equivalent fluorescence, each conjugate molecule must be shining with the equivalent of half an MESF per molecule.

This is what we have called the effective F/P ratio because it is the amount of fluorescence that you get from a conjugate. This is how we use the solution as the equalibrator to match the fluorescence between a particle standard measurement and a conjugate label itself. That's the whole thing in theory.

Now these measurements can be made fairly directly in a cuvette. They are not intended with columns of trying to read a micro-particle suspension. There are lots of things about that conjugate measurement that come, up but those at least in theory are solutions with that measurement.

The other thing that came out of the

meeting was the NCCLS subcommittee which was convened officially in 1999 or 2000. NCCLS is a long standing clearinghouse for tackling the kinds of problems that we're talking about. A couple of years ago, Gerry, in the official regulatory sense there is now some equivalence of an NCCLS document. The FDA is allowed to use NCCLS approved guidelines as the basis for their evaluation of products. Is that correct, Gerry?

DR. MARTI: Yes.

DR. VOGT: So that was an attempt to streamline the FDA process so that they didn't have to ask their submitters to reinvent the things that NCCLS had gone through. So, there is actually some, I don't want to say exactly teeth, but there is some bite to having an NCCLS guideline.

I looked at our guideline and it said "DRAFT" to be distributed in summer of 2003. I thought I'm right on time and then I remembered that I changed that before I sent it to Howard so that's why it's updated.

It's really true. I have in my hand chapters nine out of ten, chapters one through nine, out of ten, that is actually in the hands of NCCLS now and out of my hands. We will get chapter

ten to them and have this out to the Committee members and advisors next week. The voting draft will undergo one quick round of review. I think we will get voted out. It had already been reviewed extensively by the membership. Those comments have been incorporated and it should have been out here about a year ago. It will be to you all who are aware of this process and out for public comment in the summer. We would ask certainly everyone in this room to be part of that public comment process.

Then it will go for three months and then it comes back to the committee. It gets revised based on public comments. Then it spends a year as a proposed guideline and after that becomes the approved guideline. That's the process.

Here is the title page just to prove that it really is. This is the NCCLS boilerplate stuff. These are the ten chapters. There has been some revision. It's actually shorter now than it was before even though more material has been put on it. We try to go through the concepts. We actually do use this as somewhat more of a teaching document than traditional NCCLS documents have been. We fought the battle to get that. I would

like to see it used that way.

You can see the major topics that were in discussion here are in the chapters there. I want to go right to chapter nine, Quantitative Ligand-Finding Assays Using Fluorochrome Ligand Conjugates. That's what we are talking about here. What we've just sent in chapter nine a few days to Lois, our liaison at NCCLS, and here is what we sent her in outline form.

The first thing is a short discussion of what we're talking about how we get this equivalent fluorescence and binding values. Then we talk about the molecular properties that influence the binding and the fluorescence measurement. There are the fluorescence properties, of course, and we talked about this effective F/P ratio. There are binding properties in the conjugates.

There is the issue of having micro heterogeneity in most of the conventional fluorochrome ligand conjugates we use. That heterogeneity is with respect to the number of fluorochrome molecules per conjugate molecule. We can get an average value but of course that can vary across a distribution. It could be a Y

distribution. Then, that in turn can affect the binding properties of those molecular subspecies. That can be a problem. I suspect that is a problem. I suspect that's one of the things that's causing some of these anomalies.

That led us to go back and look at the recent use and very effective use of unimolar fluorochrome ligand conjugates. Here I'm talking about the one-to-one PE conjugates. We'll hear much more from Lance and from Norm about this and the practical use and we look forward to that.

But it also reminded me going back many years ago to the papers that were published by Bob Ashcroft and Ron Chatalet where they did epidermal growth factor binding. I remember the big deal Bob made or Ron, one of the two, was that none of those experiments worked until they took their FITC EGF and purified it on an HPLC to get unimolar FITC conjugates. Then all of that stuff with their isobarometric titration techniques worked.

So I think the notion of unimolar conjugates, or well defined conjugates which at the moment are not economically justified in the realm of manufacturers, is going to cost more money to make those kinds of conjugates and characterize

them and so on. That's one of the market force driving things that we have to get into that cycle of when does it become reasonable for a company to attack that problem. Only when we start buying the reagents is the answer.

Then we talked about exactly how you would go about quantifying the number of conjugates, the binding capacity. You can do it indirectly through an MESF calibration. You can do directly through binding units. In there we mention the fact that when you are looking for binding unit calibrators that there has been some recent success using biologic calibrators. Ultimately we would like to see standards that are easier to use than biologic calibrators.

Sources of variability. This is going to be the bugaboo. You are going to have differences in the fluorochrome ligand conjugates. If we get effective FP values put on them, are they authentic? Are they traceable to reference solutions? If we are using binding values that have been assigned to calibrators, whether they are micro-beads or biologic calibrators such as the CD4 cell, are those values we are presuming correct?

What about the actual authentity and

saturability of the binding? There are some misbehaviors in these systems. And what about the multiple interactions between the conjugates and binding targets, particularly when you get to multiple things. That's the shopping list of problems that come up.

And, then, finally, I think some of you alluded to this a little earlier and I was glad to hear it, we might actually want to get QFC to the point where we are measuring affinity constants. One thing you can do at a multiplex setup and there is a lot of stuff about multiplex that you can always analyze. You can also measure one analyte much more carefully if for instance you use microbeads that are labeled with different fluorescent barcodes that have different binding capacities. You do a checkerboard titration where you have those in one dimension and in the other dimension you put differing concentrations of your conjugate using this Ashcroft-Chatalet technique, you can bootstrap a scattered plot and get an affinity constant. If it's a straight line and well-behaved then you are probably dealing with characterizable binding. If it's not, then you are probably not.

I think there's a whole other dimension, literally, that we could apply a quantitative flow to once we get these standards. These things might be as important as the MESF per cell or ABC per cell. That's it. I'm sorry this has taken so long and we're still not quite finished. By the end of the year, this guideline will be on the streets ready to use and I hope that it will be useful in this process.

DR. FISCHER: So the NIST things that you are referring to, just the SRM, is available right now. The RM is coming soon.

DR. VOGT: Yes, and that of course is fluorescein. Fluorescein is still useful but in addition it was a model system to look at process in general and in particular look at a fluorochrome that had a lot of environmental sensitivity to it. The idea was that you would have to have micro particles that were reflective of their environment so that they could probably calibrate the cells within that same environment.

DR. FISCHER: Are there going to ones for the other eight colors?

DR. VOGT: Yes, for the other colors. Dolph, would you want the same thing where you

think the NIST program will be competitive with respect to the other fluorochromes?

DR. GAIGOLOS: That pretty much depends on what groups like this say. We're a supporting lab. Do you want a PE standard? I hope not.

DR. VOGT: I would love to see a PE calibration standard come out of this meeting because I think Mary Alice's examples of PE. I changed all my green colors to orange for this meeting. One of the things when we break out and talk about laboratory exercises between now and the fall meeting is we would like to work with folks who are interested in this and headed in that direction.

There are a variety of calibrators out there that can be applied to PE. I don't think we're that far away from getting consistent measurements. But nobody has actually tried to do that in a totally focused way although Lance and the group at UCLA certainly have made great progress. We will see where we stand and where we can go with Phycoerythrin. After that I'll retire.

DR. FISCHER: That actually does become a very key point because as we all know what is the one molecule everybody likes to use for that? And

the reason that you use it is because it's so good. That tends to be the one that also is the one that all clinical people want you to use for all of the markers that they're looking at for everything because they can say yes, it's the brightest one. We use FITC but it's only one of nine. The PE is very important for us because of the fact that we do a lot of intercellular staining.

DR. VOGT: Ken, can you give us any little heads-up on where NIST and PE might be?

DR. COLE: Well, we're just starting. We have an interest in assays for Homeland Security. That's what brought me into this. I don't have a lot of experience with flow cytometry. But antibody-based assays are something that we are just starting to work on. So any suggestions we're open to hear.

DR. DAVIS: Along the lines of trying to be practical with this, is this something we can add to the list as part of the protocol which would be to compare existing calibrators that are out there commercially to what NIST has? That kind of thing. So it's a request for the labs to come back and look at these and do we all get the same interrelationship. That could be of value.

DR. FISCHER: Bob, you've actually brought up something while you were talking about NIST traceable standards. Now having spent more than one year in a private company and having had an accrued GOP and GMP and all those kind of regulations, the fact that you have to have the ability to use mistraceable standards for a lot of the equipment that we use when it came down to the flow cytometer, they weren't available.

Here we were trying to follow all the regulations and we asked what do we use for a flow cytometer. I was told that there isn't anything that is the one approved method for building flow for, being something as simple as calibrant. Now we have the CyAn and that's nine colors.

DR. VOGT: Well, we won't have nine colors tomorrow.

DR. FISCHER: I'm a realist. I know that these things take time. The fact I didn't see anything concrete come out of the last meeting was only because the stuff was being done in such small increments. I guess eventually it all comes out. Maybe it isn't publicized well enough for those of us out in the flow community. Look, this was done and you need to go look at these things if you want

to do quantitative flow. Maybe that should be one of the keys out of this meeting too. We should get the information out there not just to the 30 of us who are in this room, but to the 3,000 people who are out there who are running flow labs.

DR. VOGT: There was a prolonged ramp-up period. Part of that was just due to me personally. At NIST there's always a ramp-up period. But Dolph and his colleagues were on this solution and everything as quick as could be. So it's my impression that we can move farther faster now. If all 200 of those fluorescein standards were gone in a couple of months, that's going to be the biggest mess. Is that right, Dolph, that the popularity and marketability of that standard would influence the thinking in NIST?

DR. GAIGOLOS: Probably not but if it's not popular, it will.

DR. STETLER-STEVENS: So we have two issue items that have mentioned as actions, one to compare standards and one to publicize what's been done.

DR. VOGT: Right and, Bruce, absolutely that's what we want to do. Between now and the fall, if we can do some scratch studies which are

some things between labs that are interested in doing this both levels of cytofluorometry and cuvette fluorometry for those that are interested. There are those here who are.

They can work with Dolph and Ken on this and get them engaged in that initial process, then they will see how bad things are or how good things are. They can take that information and move it into a standardization program. That's a very important output of this meeting.

DR. SHAPIRO: In terms of publicity, the quantitative flow drum gets beaten pretty hard for the condition of flow cytometry and I could explain much of this stuff in English.

DR. FISCHER: Is that done yet?

DR. SHAPIRO: It will be in a couple of months. It's done. It's along side the NCCLS stuff.

DR. VOGT: Right, I was just waiting.

DR. MARTI: One other point that I think that perhaps is useful here is that the use of quantitative flow is somewhat specific in the history of the development of an assay. Where an experiment or experimental finding was just groundbreaking in the basic research lab where they

might be the experiment at least twice, on a good day, maybe three to five times, the kind of controls and standards that we need for that experiment are much different from a product that you want to license and sell between states in the U.S. The kind of stringent controls that are placed on that are much different.

Even watch the beginning of the multi-bead multi-analyte. It started out and look like it was going to be really good. Then it slowed down. Now it's picking up. It's getting its second burst. Or use the micro array. Have you ever seen two papers that had same answer yet in micro array? Talk about standardizing that. But it will come if it's going to be used to make decisions about what drug you get or you don't or whether your arm is going to be amputated or not. The kinds of controls that come on that will be much greater.

DR. STETLER-STEVENS: Abe, you're up next. Are you going to tell us what doesn't work? Where the flat tire is.

DR. SCHWARTZ: I might not be invited to any more meetings after I speak today unless you want to make yourself feel good. I can do that for

you also. I have no vested interest now, as most of you know. So I would like to make some observations because I am working as a NIST associate researcher. I've been involved with Dolph and the colleagues. I've done the MESFs and ABCs for about 20 years. They have made some significant improvements on what I've done because they have the instrumentation and the knowledge on the formalization of fluorescence.

There are two or three things that have been improved and will make this thing work even better than it has is one, they have the fluorometer that has holographic filters. This allows you to do the whole intubation of the emission spectrum like Bob mentioned. It gets rid of all the background to where two Million beads per Milliliter looks like water in terms of background scatter.

Before that when I started back then it was 500,000 MESFs. It was so bad that I had to make another type of beads that had no scatter, get the assignments and make a calibration curve on the kind of beads they actually commercially use now. That's a major number one improvement they did.

Number two, is that we made a series of

beads with different extenders on it. We found out that ones that have sold for 15 years do not match spectrally with antibody-labeled cells. You will see this in the third paper. If you had a longer extension they match almost perfectly with antibody-labeled cells. That's the second improvement. You have to really characterize your calibrators to match what you are measuring.

Back to the title of the thing of what doesn't work, these conferences don't work. How many of you have been to standardization and consensus conferences? Again I've heard it. We walk away with that sounded good and nothing happens. NCCLS documents, I think is going to be a positive thing. The collaboration with NIST is also a positive thing.

The comment that was made that we should really have four or five labs really doing this because they're the only ones who are doing it, how are you going to do it without standards? Because if there's only five people buying them, nobody will spend the effort in making them. They aren't made right yet. Nobody.

For example, who buys BD and Bangs PE calibrators? Do they come up with the same

calibration curve? Do they?

DR. LENKEI: Probably not.

DR. SCHWARTZ: Anybody have hard data saying yes or no?

DR. BRAYLAN: The hard data is no.

DR. LENKEI: Univan is working on the second project shows that we can come up with it.

DR. SCHWARTZ: Are they all on top of the same line?

DR. LENKEI: Yes.

DR. SCHWARTZ: They do? Al says no. I haven't bought either of them so I don't know. I suspect that they don't yet.

PARTICIPANT: I haven't seen a lot of them.

DR. SCHWARTZ: And they haven't. These are two companies putting out standards that it doesn't even matter if they're right. They don't even agree.

DR. LENKEI: And speaking about MESF.

DR. SCHWARTZ: Correct.

DR. LENKEI: It's not about ABC.

DR. SCHWARTZ: I didn't say ABC. BD doesn't make ABC.

DR. LENKEI: The correlations?

DR. SCHWARTZ: Correlations of 99.99, but they aren't the same answer.

DR. VOGT: The assignments are definitely not the same.

DR. SCHWARTZ: Why as a community do you allow that especially when you are spending money for it?

DR. FISCHER: That's the point. In several years we haven't bought any of those because they didn't match. Because we couldn't use it from one lab to another, that was why we stopped buying them. Most of our money would talk even when the companies didn't seem to listen to them.

DR. SCHWARTZ: Why didn't they listen? Because they have to survive. I know it more than anything because that was my only products. These people have 100 other products. If people don't buy these, business-wise it doesn't matter. To this group, it is the key. If you do anything, it is to address that issue of being able to have the material that you can start answering these other questions. If you do not, don't waste your time even asking.

DR. FISCHER: Doesn't that go back to

the point of making them NIST traceable?

DR. SCHWARTZ: NIST isn't going to do it.

DR. VOGT: I'm going to disagree with that. I think we can come up with Phycoerythrin units that are traceable.

DR. SCHWARTZ: Who's we? Who's going to do it?

DR. VOGT: The people in this room.

DR. SCHWARTZ: So NIST isn't going to it and the companies aren't going to do it. You're going to get your own bead labs and make them.

DR. VOGT: No, the companies and NIST and the people in this room are going to it.

DR. SCHWARTZ: NIST doesn't have the budget. Like you said if you don't buy it, fluorescence is dead.

DR. LENKEI: I want to say something.

DR. SCHWARTZ: If you do buy it, they may try to do it but the amount of energy and effort especially making a PE particle standard is not going to happen.

DR. VOGT: I think it will. I think you're wrong. In three years, we'll have universal PE units.

DR. SCHWARTZ: It's not the units. It's the material. Who's going to make it and who's going to verify it because it's not in the program?

DR. VOGT: BD has had a product for years.

DR. SCHWARTZ: And so did Bangs.

DR. VOGT: Wait. The BD product was tethered to their conjugate. It works. It absolutely works. I don't think it's fair what you are saying.

DR. LENKEI: I want to say something. Conjugation is standardization. So if you speak about selling reagents, to sell them in standardization because I have that I didn't use that because they don't leave us with the exact pairs. But using them in the last ten years, I had a very good standardization. If we tell them, also more is standardization of reagents to get unified results in order to get better significance in all our established teacher grades.

DR. VOGT: It can be done, Abe.

DR. SCHWARTZ: It's part of the question that it can be. I don't see anybody putting the necessary resources to do it correctly.

The theory works. Dolph has formalized it and Lily to the point where even I'm convinced that the subjective thing to say is I think this works in proper and it worked for 20 years. People got the kind of the same answer. But in terms of it being really solidly based like we tried to do in North Carolina as a white paper we said his MESF theory, does anybody have a problem with it? That wasn't very satisfactory because the only problem was why are people making money on it.

DR. LAMB: Abe, part of the focus that flow labs are going to buy a standard and use it and that's what's going to drive the thing. It's not going to happen. You're absolutely right. He made an excellent point from Pharma's point of view.

If you are working with tumor-associated antigens which are now just starting to get over the hill as something that's going to be important and in targeted therapy of leukemia which is what my lab works on, when these go to Pharma, what dose of this drug and what antigen density of this cell is going to give you efficacy? Those are the companies that are going to drive this doing thousands and thousands of tumors on clinical

trials. Not at a pathology laboratory but in an industry setting.

Then perhaps the technology will get to the point where it will filter down to the individual laboratories. But you say nobody's going to buy it. I'm tell you what. I think the people will buy it.

DR. SCHWARTZ: I'm not saying they wouldn't buy it. I'm saying nobody has the will to put the resources to do to give you the material that you can use. It's a Catch-22. I tried to put as much as I could into it.

DR. MARTI: I think that industry won't pay any attention.

DR. SCHWARTZ: And NIST.

DR. PURVIS: The regulatory agencies are going to be the ones that will drive this as well as the insurance --

DR. MARTI: From my viewpoint, just because a standard doesn't exist in industry, if the market is deemed to be $200 billion, that standard will appear.

DR. SCHWARTZ: It can't until you have proven it's valuable. I've been asking for 20 years how does this save lives. Nobody has said I

can't say where it's going to save lives. Now you are coming up with some things that maybe it's important. You are not going to get the two Million assays required to use as a pressure to get somebody to take this stuff seriously to put the effort and energy and resources into it to make a thing that will standardize it.

DR. WOOD: I don't think it's quite as dismal as you have painted it because what you need to look at are the two factors involved here. It's precision and accuracy. If we can shoot for being precisely inaccurate, we can always correct later on. Our problem right now is we need precision. So it doesn't matter whether you get two different numbers. You can argue later on as to which number is more accurate. But, if the precision isn't there you can never argue that.

DR. SCHWARTZ: The precision is getting the same number if it's wrong or right.

DR. WOOD: No, accuracy is getting the same number. Precision is a typical manufacturing problem.

DR. SCHWARTZ: Precision is getting some number that is repeatable by various labs.

DR. WOOD: Right.

DR. SCHWARTZ: It may be right or wrong.

DR. WOOD: Whether it's right or wrong, it's the same number. That's what is important.

DR. SCHWARTZ: That's one number whatever it is.

DR. PURVIS: The same assignments on these beads or you have to have an FP that corrects four of the assignments on the BD.

DR. SCHWARTZ: Because in general we all have to get the same answer whether it's right or wrong. That's what precision is.

DR. WOOD: Correct.

DR. SCHWARTZ: We're not doing that. We have two products out there getting two different answers.

DR. BRAYLAN: That's accuracy.

DR. SCHWARTZ: Both may be wrong but they are different.

DR. BRAYLAN: Right but within each system is repetition.

DR. SCHWARTZ: If you are going to have one disease and you are using one system and they are using another and it's two different answers you can't talk to each other.

DR. VOGT: In these settings a better word than accuracy is bias because it actually is more indicative of what's going on. The difference between precision and accuracy becomes very flaky at some point. What's happened is in flow cytometry generally you have very precise measurements because you are counting so many events. So you can see small differences between things and that is in bias. So there is bias between the PE calibrators, but there is good precision within the use of any particular PE calibrator.

DR. SCHWARTZ: The more manufacturers and standards you have that are getting different answers, it's lucky you only have two that are doing it.

DR. VOGT: That looks like kept work.

DR. SHAPIRO: The guy who shot the red painted bullets is accurate but not precise. The guy who shoot the green painted is precise but not accurate.

DR. SCHWARTZ: But what happens when you have different groups here that gives you a couple of answers. Which one do you believe?

DR. VOGT: Then goes back to what Jim

said which is that you can always move to the center if you have a tight cluster anywhere that's in the measurement space.

DR. SCHWARTZ: But it may not even be more important to get to the center.

DR. MARTI: Remember when we started CD34 in U.S., Canada and Western Europe. The variation on a CD34 measurement published was plus or minus 1000 percent. The next thing that happened in the process was let's take a bunch of labs and have a standard method, and you and your lab and your wisdom and your extensive experience, you compare your local method to the standard reference and we're going to do that four times. Guess what happens? They couldn't complete the experiment. After the second mail out, everybody was using the reference method.

DR. VOGT: They broke the code.

DR. MARTI: I don't know about that. But the moral of the story is that these two can move together, or they can move toward the center. The whole idea of standardization is to try to and define what the magnitude of the variation is.

Once you know what that variation is then you can take it apart and see what it is. We

generally agree up to this point in time that the variation isn't due to the instrumentation except sometimes we don't know that. We don't know that the instrument's been validated. But when the instrument is validated then the instrument is removed from the equation. I think that's been true in the past. I'm not so sure now with this new generation of instruments whether that's true because it hasn't been tested.

DR. FISCHER: And in the long run don't we need somebody to tell us where the bull's-eye is? That's where NIST or somebody come in because they have to tell you where the bull's-eye because we can all end up outside of the target completely. Yes, we'll be precise and we'll all in some ways be wrong, but until somebody tells us where the right spot is, we'll all think we're right anyway.

DR. MARTI: In retrospect the fact that when we were all getting the wrong answer on CD34, at least on unmobilized peripheral blood two to three or five CD34 positive cells per microliter, it probably was right.

DR. MUIRHEAD: There are two issues. One is the bias issue that Bob brought up. The second is the perception issue. That's an issue in

a larger community. In this room or the people that have been thinking about these problems, I think they would agree with a statement that says as long as we know what the biases are, as long as people who are using a given system can get consistent results among the group that's using that system, then we decide there's a way to account for that bias and know what it is. Fine. Then we can start preparing the process.

But to the outside community, then, they are thinking about this, the problem that says I see a group using this system that's getting what looks like a completely different set of answers. That's a perception problem. That's one of the issues that says wait a minute, I don't want to have anything to do with this because the experts can't even get comparable results. So you have to deal with both of those issues.

DR. SCHWARTZ: The biggest concern I have is the material that is certified is not going to be available because there's not the support to do that. Even for NIST, that program is doing these two things but somebody asks what's after that.

DR. MARTI: Standards. It is a big

glorious word. But you are never going to get an RO1 funded from NIH on standards. That's the first thing. The second thing is where standards develop and it's supposed to be a big thing at the FDA, I think that every time I write it down on my CD or on my plans for the year, that automatically means negative dollars. That's a reality. I agree with you if there isn't a standard that can't be marketed and properly made on the effort that we need to make it, that's a problem.

DR. SCHWARTZ: That's your biggest problem. The rest of these things don't mean anything unless you have the material to hang your hat on to deal with these other problems. I'm not saying they are not important. They are very important. But you have to have the standards set up first, it's worthless.

DR. MARTI: I guess in order to pull ourselves out of this severe depressive area where you are.

DR. SCHWARTZ: I'm not depressed.

DR. MARTI: The dismal state that you're painting, we can do some things about that with the materials that we have. God forbid even if we have to go back that furaldehyde fixed

chicken red cells or thymus nuclei.

DR. HOFFMAN: We do have a lot of resources. We have a lot of capabilities. Let me just say something. I don't think we developed quantiBRITE because we thought we were going to make a lot of money selling quantiBRITE. We developed quantiBRITE because we thought the presence of quantitation was going to be in the future a very useful and hopefully profitable kind of business.

We published how we assigned the values. We published what the molecular weights we assumed from literature values for the PE. We published what we assumed from literature documents the extinction coefficient was. We published all the details about holocene, two inventive methods and we assigned the values and got us some results.

If there are biases there, it's likely coming from the assumption of what the molecular weight was or what the extinction coefficient was. There are resources around the world that know all about hydroproteins. We're in contact with some of those and maybe others. Even if NIST doesn't develop a standard for PE, companies that are interested in developing, manufacturing bead

standards have resources at NIST.

BD is interested and we're working with NIST, not necessarily because we expect them to do all the work, but because they are a valued resource. They have this incredible fluorometer. If we can get groups that can agree on things about PE and there are proteins and biologics and there are variations from one kind of bacterium that's making it to another, whatever source of PE that a particular manufacturer is using for their conjugates, we have resources at NIST. We may have other resources in this room. They've been used to come a more complete understanding and assignment of agreed upon values.

Maybe we're not going to have two systems that are going to give exactly the same results, but within a system a PE conjugate, a critical source of PE, we will be able to get consistent results. Then knowing what the biases between those two are, hopefully we can maybe know what it is.

DR. SCHWARTZ: I'm not saying it can't be solved. I'm saying that there's not enough serious focus on getting the resources to the people to be able to do it.

DR. HOFFMAN: I'm just following up with some resources. I'm not suggesting that more than NIST is actually going to want to do it but I certainly hope so.

DR. SHAPIRO: Again the process exists. We know if you have a fluorometer that's appropriately designed you can do these measurements whether they're your beads or anybody's beads. You have a solution. But if you have a label and you have beads and you have the fluorometer set up with the appropriate flow, you can do this. Once there is one machine around, the initial investment is in the first machine. While the filters may be expensive, they're not that expensive. So basically it's fairly easy to implement this process.

DR. SCHWARTZ: NIST's role is to find out the methodology so other people can do it.

DR. SHAPIRO: Right.

DR. SCHWARTZ: And they've done that.

DR. VOGT: The thing is, Abe, even after all that is settled, that's not the biggest problem Mary Alice has. The fluorescence properties and the calibration of the fluorescence scale are tractable problems. The use of

phycoerythrin conjugates for staining cells will present perhaps some intractable problems but they will have to be worked out on a case-by-case basis.

That's what I'm more worried about than our ability to get a handle on PE fluorescence. I think we can handle on that, but I'm not sure that we're going to be able to get a handle on every single phycoerythrin conjugate that's made by every single manufacturer. It's still going to be up to the manufacturers to provide conjugates that are characterized in a way that work in their systems.

DR. SCHWARTZ: Let me just part with just one comment with what will work. It is what Norm Purvis`s approach has been for the last half dozen years or so. ABC binding beads will work in the hands of somebody that has the time and energy and effort to do them. That's not most of us, including even myself. I would have to have specialized technicians to be able to do it to get consistent things as best I could do it.

What is practical and again Bob was saying on this effective F/P ratio is if we can develop any MESF beads where everyone agrees no matter what their source they give you the same calibration line because that's what you want and

then the manufacturers who make the reagents can put on that bottle because if you have to try to do it yourself, you're in a hell of a mess and it's a waste of your time and you would be willing to pay a few more dollars if that F/P ration is on the bottle. You divide that by whatever you have for MESFs and you will get consistent answers. Whether they are right or wrong, I really don't care and neither do MDs. As long as they have an answer, they can make a decision about it that's consistent. That's all we want in this room.

DR. LENKEI: We can't rely on each company that the figures will be correct because this is a big problem.

DR. SCHWARTZ: It will be correct because hopefully NIST or somebody like that will go into a document like NCCLS and say this is how you assign the number to your antibody. You can do it with a fluorometer. That's the easy one. The problem there is do you know the concentration of the antibody that's going in to get that particular fluorescence. That should be the key push for here in terms of trying to develop something that you can get answers, that you could interpret or misinterpret because of these other additional

problems.

DR. PURVIS: This removes the need to also those unimolar antibodies.

DR. VOGT: It might or it might not, Norm.

DR. PURVIS: It depends on how good the conjugate is. As long as the antibodies have not been over-conjugated where we have quenching or have affected the binding.

DR. VOGT: I'm wondering if that's ever going to be possible to insure.

DR. SCHWARTZ: It will because it's tested before they sell it.

DR. VOGT: But again every cell is different. Can you guarantee me that just because it didn't interfere with a complex set of markers in one tumor that it won't interfere with a complex set of markers in another tumor?

DR. SCHWARTZ: No.

DR. VOGT: Nobody in this room would guarantee that.

DR. PURVIS: I think a part of that is going through and doing the proper qualifications on the antibody that you're interested in evaluating. That's simple biology.

DR. VOGT: There's a lot of biology.

DR. SCHWARTZ: And on the bottle it will say that this can be used for such and such a tumor.

DR. PURVIS: They will not put that information on the bottle.

DR. SHAPIRO: Nobody can afford to do that.

DR. SCHWARTZ: That's my two cents.

DR. LENKEI: One point we should look at is FITC or functions specifically to the predicament of fluorocarbon for conjugate.

DR. MARTI: Just go back one step. I may have had a transient temporal lobe seizure there. What was the consensus of the group about how you want a label to read on a vial regarding a fluorescent ligand conjugate?

DR. SCHWARTZ: The effect of that would be how many MESFs per antibodies and divide that number into the MESFs of your cell which is a more stable type of measurement. You get the binding antibodies.

DR. BRAYLAN: So how difficult is it for the manufacturers?

DR. SCHWARTZ: Simple. He does it

every week.

DR. BRAYLAN: If that is simple then this should be done.

DR. PURVIS: For the manufacturers, it should be even more simple because at the time that they do the conjugation and antibody verification they know the concentrations.

DR. SCHWARTZ: Right, they know what the concentrations are. It's very hard for them because they put other proteins in there and you can't tell what the real concentration is.

DR. VOGT: I want to emphasize one point that Bob made. This stuff that they did was all published. It's all in public domain. That's really important because if we have a bunch of manufacturers that do their own method of doing this we will have biases. We'll probably have some biases anyhow.

When stuff appears in the open literature and everyone can look and see how it's done, and there some conflict here with the ability and need to make money off of things that may be proprietary. There will always some difficulty in balancing those things.

But the BD methods are published.

That's a very important thing. That allows us in the NCCLS guidelines to refer to the BD method which we could never do referring to them as BD methods, but we can certainly refer to the papers that appear in the open literature. One thing that I would like to encourage all the manufacturers to do is publish, publish, publish.

DR. PURVIS: Can we not use what NIST has developed here for procedure and implement that as a consistent way to measure for the manufacturers to actually use this? This is going to be published.

DR. VOGT: I think what Dolph has published for the assignment of values and so on is going to be used in a few laboratories. But the assignment of the effect of FP or whatever you want to call it is trickier. If you look at what was primarily Ken Davis and his group did at BD in working that up, it's a complex thing. It worked real well for one marker and then it took more work to get it to the CD38 zone in time to tell us about it.

DR. MARTI: But it's safe to say that there is a desirability and it would be desirable to have something about what approximates an

effective F/P ratio on the label of the antibody ligand conjugate.

DR. VOGT: Absolutely.

DR. MARTI: Going once, going twice, gone. That's something that could be discussed with the manufacturers and get their input to see what they think. Remember that in this room you all take what you read on a label pretty much for granted. But what's on a label from a regulatory standpoint, that's looked at as how much did that label cost. How much research and development went into that label. Did it cost a half a Million dollars to guarantee what was in that bottle?

Industry likes to not to have to put much on that label because the less they have to put on, the less costly it is. As simple as this sounds, I would still keep open to the fact that there might be some dollar amount associated with putting that on a label.

DR. SCHWARTZ: If I had that on a labeled antibody, I would pay extra for it because I wouldn't have to do it.

DR. MARTI: If the cost of an analyzed specific reagent would go up 10 percent, would you pay for this?

DR. McCOY: As long as the Medicare reimbursement goes up by 10 percent.

DR. MARTI: Oh boy, that's another meeting.

DR. BRAYLAN: As long as you get paid either by research or also by clinical policy, I would say that I would be very happy to pay for CD 20 or CD 22 because I know there is clinical application. Otherwise how are we to pay?

DR. FISCHER: I don't think you get it on every reagent. But you want to get it on the main T-cell and the main B-cell quantitative antibodies.

DR. SCHWARTZ: A new classification is what we are talking about.

DR. VOGT: Since we are talking about phycoerythrin reagents dominantly here, I want to go back this unimolar business. If you look at BD papers apparently there is not enough quenching upon conjugation to alter the fluorescence yield of phycoerythrin on an antibody molecule. So the F/P ratio of unimolar conjugates is one. It may be 1.0. It may be 0.9. It may be 1.1 but it's not 1.5 and it's not 0.5.

Gel filtration is easy. I don't know

exactly how you purify your antibodies, but when you take something that's 150,000 and add something that's twice as big to it and you need to separate that from something that's 150,000 with two things that are twice as big on it, that's a pretty easy problem in biochemistry.

That's probably easier than figuring out the effective F/P ratio and knowing that you are not altering binding properties by having heterogeneity of your conjugate phycoerythrin ratios. So I actually think unimolar conjugates for phycoerythrin give us both. They give us a constant effect in equivalent fluorescence and they give us constant binding properties.

*DR. SCHWARTZ: But, I don't think that's material.

DR. VOGT: That's the price you may have to pay.

DR. HOFFMAN: We sell some one-to-one conjugates. The price is higher because the yield is less.

DR. LAMB: You have to buy in volume.

DR. VOGT: How much higher is it? Do you know? I actually don't know.

DR. HOFFMAN: I think it's 20 percent

or something. It's not twice as much.

DR. LAMB: Bob, the deal is I have bought CD69 one-to-one. You can't order a bottle of it. You have to order a lot.

DR. SCHWARTZ: $5,000 worth.

DR. LAMB: Which they will make for you for about $5,000 and then you have that in your lab.

DR. HSI: CD30 right now is five times as much as the regular CD reagent.

DR. SCHWARTZ: But you don't have to be limited to phycoerythrin if you do it with fluorometry, you can do it on fluorescein or any other fluorochrome.

DR. LENKEI: We can show you the very inch variation and I don't think any company would like to tell you exactly the difference**

DR. SCHWARTZ: Do you mean if they go back to fluorescein?

DR. LENKEI: Not only that you can submit antibodies since they test fluorescein for -- - is also part of the company's confidence not to show you exactly.

DR. SCHWARTZ: When I measured these things by the ABC method for fluorescein, I was

always coming out 0.7, 0.6 for fluorescein almost across the corporations. So it's not any problem.

DR. LENKEI: I will tell you. I had an amazing range in the beta monoclonal from 6.1 fluorescein protein ratio to 9.0. I almost was going to correct all my figures because it was not as effective.

DR. SCHWARTZ: Was that on the label?

DR. LENKEI: It was a fluorescein label. But I heard from them when I saw this.

DR. SCHWARTZ: That's how much they put in. That doesn't mean how much fixed.

DR. LENKEI: Yes, but it was a bed monoclonal-- it showed because it had nine and inoxidated part of my monoclone.

DR. VOGT: I was going to say you get nine FITCs on a antibody. Let's go back to Ashcroft paper. When they tried to do that experiment with conventional epidermal growth factor fluorescenated ligand, it didn't work. When they purified it and got unimolar, it worked beautifully.

I'm not convinced that the biggest problems here are fluorescence. In fact I'm firmly convinced the biggest problems here are binding. I

don't think we're going to get around binding problems by getting effective F/P ratios. We may get around the binding problems by having uniform conjugate reagents.

DR. SCHWARTZ: Not only PE.

DR. SHAPIRO: Probably not PE because as along as we have the work horse laser beam 488 and as long as we want to do seven colors up to 488B then we have PE tandems, quantifying is going to be impossible. If you want to do a quantitative measurement with PE, then you really have to start thinking about gating with antibodies that are excited at other wavelengths, whether its in red or violet or UV where you don't have the spectrum crossed off with PE.

That's giving you maybe four antibodies to gain on it. If you want data on more antibodies than that, then maybe you start thinking about considering the ABC reagent for your quantitation even though it's not going to be as bright as a PE reagent. There is no question that if you are doing PE with six other colors, the cost of it is just ridiculous.

DR. TAMUL: I'd like to second that and also to mention that the manufacturers would like

to have the standards just as much as everybody else would. We need something else to go on as well.

DR. MARTI: Mary Alice, before we go on to the next presentation, I would like to make a final comment on Abe's presentation. Abe, one of the really wonderful things that you've done was to use the consensus process to establish a set of performance characteristics of standards. You primarily did that with one of our colleagues who is not here today, Frank Mandy, from Health Canada. That certainly is a big torchlight to have that concept analysis.

DR. SCHWARTZ: That and the window of analysis.

DR. MARTI: I would agree with that too.

DR. LENKEI: It's the biggest thing which we have.

DR. MARTI: That still needs to be tested a little bit further. It needs to be tested in beta setting and not just Western Europe.

DR. LENKEI: We have published the results.

DR. STETLER-STEVENS: I've had a

request for a five minute break. Excellent. Have something to drink, go to the bathroom. Five minutes though. Real quick. Off the record.

(Whereupon, the foregoing matter went off the record at 10:15 a.m. and went back on the record at 10:32 a.m.)

DR. VOGT: Well, Jim, there's actually a very good convergence on those measurements over a 15-year period.

DR. WOOD: Well, what I'm saying is to go from one manufacturer to the other, it's going to be important in order to get the precision and reproducibility, as to work within a system. That is you're going to have to have consistency in the antibody, consistency in the dye that you're using and develop a whole system around that for calibration. And then to go from manufacturer to manufacturer, you're going to have to have fudge factors, for these calibration factors to go across.

DR. FISCHER: But you have to go back to having a standard to base all the fudge factors on, right?

DR. WOOD: Well, but then you go back

to identifying what CD4 is, for example and say which antibody because you talk about what's a bad antibody. Identifying and making sure that it's really identifying the epitope the way that it should do it.

DR. PURVIS: I'll throw another problem into the mix. If you have the same clone from multiple vendors.

DR. VOGT: You mean the same antibody?

DR. PURVIS: Same clone, same clone.

DR. VOGT: Okay.

DR. PURVIS: If I do a PE conjugation to it or buy them all in PE's, I get all kinds of different results. The F/P's are different and that is where I need that F/P to be able to take care of it. Even if I think, based on what I'm seeing, Prozyme, I went out on their website and I talked about PE's degradation, in the conjugation process itself, even if you gave me unimolar PE conjugates, I'm still going to see differences because the effect of F/P is not one.

DR. WOOD: Just to talk about the issue with phycoerythrin, if we're trying to take different manufacturers with different clones or different versions of PE, and you go to, say, using

the Alexa dyes, what you've done is you've now gone to a single manufacturer again in terms of identifying an Alexa dye, that dye, and you identify exactly what it is.

DR. SCHWARTZ: Fluorescein is fluorescein. Alexa is Alexa.

DR. WOOD: What you're doing though is you now reduce the variability of the system.

DR. PURVIS: Yes.

DR. WOOD: And that's what I'd like to see. Naturally when you do that, then your overall precision is going to go up. That was my main point to make in the whole PE thing. Yes, it's great. It's bright. But I -- we are capable of doing it now with the new instrumentation, sensitivities that we have, the resolution, the linearities to be able to use FITC without any problems to quantitate on most of our antigen systems that we're interested in doing our quantitation of. I think the same thing would be true for the Alexas and other dyes.

DR. VOGT: There is another approach to this that I truly believe would work and this is something Abe and I talked about years ago. His original system was get the MESF curve and get the

single quantum simply cellular population with a known binding capacity and do your normalization, do your one point, the same as the CD4, except it's quantum simply cellular bead rather than a CD4 cell.

The problem with that system is there is no universal binding capacity that you can assign to the quantum simply cellular beads. Even from lot to lot in the same manufacturer, the same fluorochrome, there's variance. However, it is possible to calibrate those beads on each lot of antibody using, for instance, the isoparametric titration or using Howard's cheap and quick supernatant redepletion method or whatever.

So you actually could provide people with a matched pair of your conjugate and a quantum simply cellular type of bead, a capture bead, which had been calibrated to that conjugate to that lot. You put that on your flow cytometer and read it off in the MESF curve, you get the effect of F/P ratio in your lab for that lot, that bottle of conjugate.

The only thing you have to worry about is whether there had been some degradation in the binding properties in the quantum simply cellular

bead between the time that you opened the bottle and ran it from the time of the seal-up to begin with. That system would work but you have to have independent lot by lot quantification of the binding capacity of the capture bead for that particular conjugate. That's the closest thing to a universal system you could get.

DR. LENKEI: Well, do you remember the studies that even now, the most important thing is how you conjugate that antibody on the bead? And it has been a lot of problems and we know about that. The last protocol they were much better because they were taking into consideration the amounts. So we had a lot of problems from the practical point of view.

I want to stress here that what you have, I am talking about clinical applications because the subject of our meeting theoretically, it's very good to go farther for the next case. But for this case, I guess we had problems.

DR. SCHWARTZ: If we took what Bob said to try to make it practical for clinical people where they don't have to do a whole bunch of nonsense to get the quantum simply cellulars pre-stained by the manufacturer - and I didn't have

enough money to do it properly and I couldn't get anybody interested in it at that point - and if you pre-stain it and the manufacturer does the assignments and leaves it swimming around in the antibody, then you take a drop out and run it, you will have a calibration curve which will be accurate.

DR. LENKEI: Yes, and I can profess that in our second experiment of the task force, you prepare the reagents last and they were stable for one year. They have exactly the same number, the same intensity when we had all the same application for one year. All the peaks. And it was very good but impractical when it was the monoclonal antibody, they were conjugated by Abe. They were kept in the same conditions in my laboratory. They were distributed to eight laboratories in Europe each month, and so on. Then the variance was very low with quantum simply cellular. But in clinical application in many laboratories, we had a lot of errors.

DR. SCHWARTZ: The clinical people cannot do that work themselves. It has to be done by someone who says all right, I want to tell the manufacturer and sell this thing, that has the

right equipment and knows the problems with binding.

Essentially they solved them and sell you something that will give you the same calibration line, just essentially like the quantiBRITE does or the hard dyed beads or whatever but that's a big responsibility for the manufacturers. It really cannot be done by I've given you a protocol and you try to follow it. You do not have the time and experience to do it reproducibly.

DR. FISCHER: We're the hands. I mean, most clinical labs are overworked as it is now.

DR. SCHWARTZ: They shouldn't have to be making the standards.

DR. QUINTANA: In both your method and Bob's neither one is going to come from a bead manufacturer.

DR. SCHWARTZ: It has to come from an antibody.

DR. QUINTANA: And an antibody manufacturer is not going to want to take beads and sell them with antibodies.

DR. SCHWARTZ: Why not?

DR. QUINTANA: The same reason a bead

manufacturer doesn't want to buy --

DR. SCHWARTZ: Why not? I mean, they could buy your beads --

DR. D'HAUTCOURT: My opinion is that in my experience are able to validate certain calibrator and this is a good point of quantitation in the future. Before trying to have something that is consistent, first we must focus on the clinical application because they show clearly that the design of the system and this is so obvious that we can validate the stability of the calibrator. This is the work in the opposite sense and this is for me a good move that quantitation is useful in clinical application.

The problem is that if we try to explain everything like biology, measurement and so on, we produce so many complications in the system that most of the clinical labs have no more interest in it.

DR. SCHWARTZ: The clinical labs should not have to deal with any of that. They take something out, they get a calibration curve, they run a cell against it, and they get the same answer.

DR. PURVIS: One calibration material

for FITC or PE. This is a very simple process that BD's works by. So is yours if you have a proper F/P ratio. It is a very simple process. It can be reliably implemented in anybody's lab so that it takes a lot of the variability out.

DR. SCHWARTZ: And it shouldn't be done with binding beads. It should be done with a fluorometer and that makes everything straightforward and simple by the manufacturer of the antibodies and write that on the bottle.

DR. MARTI: Kathy is trying to say something.

DR. MUIRHEAD: I'm sure some combination of the manufacturers in here must have thought about it. One of the benefits of the CD4 method is that Mother Nature gives you a reasonably well controlled standard reference material in the form of the CD4 lymph set. Goodness knows we can engineer every other kind of cell. Are there ways of engineering stabilized cellular material that could be used?

DR. SCHWARTZ: Poncelet didn't find out what the CD4 was on cell lines.

DR. MUIRHEAD: Those could then be used across antibodies. It's a different problem but I

mean somebody must have either thought about it and tried it and decided not to do it.

DR. VOGT: Kathy, Bill Caldwell did that years ago with the B-cell line. He tried to sell it to Coulter and they weren't interested at the time. This is like early on.

DR. MUIRHEAD: This is where we come back to Jean-Luc's question. This is a chicken and an egg. We can imagine all of these things. We can probably even imagine how to build some of them but it's going to cost either time in the clinical lab, which nobody has, or money on the part of the manufacturer and therefore, on the part of the purchaser. Where's the applications that show that it's worth somebody's time to either manufacture the right reagents because the lab is going to be able to get reimbursed for it? It really is a chicken and egg thing. You have to pick some way to stop it.

DR. LAMB: You're right. It goes back to what Gerry said earlier about the FDA's requirement. Abe, the reason I quit buying your beads is because although it is a nice exercise, we sit down and we say is this on the CAP checklist. No. Is this a requirement of NCCLS document 1852

or 53? No. Is our position going to give a red flag of what MESF? I tell them a CD19, CD10 kappa cell is no. Then we look around the table, why in the heck are we doing this? It's costing us this amount of money and nothing is happening, so it's gone.

And that's the reason. In order to get that back, the person that is going to drive that is either going to be a regulator or it's going to be an oncologist who says, if you're talking about tumors, I need these data to treat this patient.It can't come.

DR. MARTI: Jack?

DR. QUINTANA: Several years ago there were two different products that we developed over the years. One was a stabilized control cell and lots of quantitation data was generated on the markers for that product. When the product went IBP putting numbers on there, FDA was going to have some issues with that. So we collected lots of years of data on knowing the stability of four or five years of the stability of the lyophilized product in terms of the expression of the markers at least to have a relative level. We generated that data. It doesn't apply.

And we've also looked at it with the new product that's been developed which is the Immunotrol which is basically a whole blood stabilizing product that actually could be put through staining in a preparative process. And data has been collected that shows stability of those. It's not to the level of course of the lyophilized cells, but not all markers can be maintained to the same level.

For a lot of the markers, yes, the data exists and it can be done, but it would require that we have to go back and on a lot by lot basis which can be done and assign an outside value in terms of the binding of an antibody.

But to Norman's point, the issue is the binding of the antibody numbers you can say it can be referenced to a specific clone. But when you look at other clones, you have to go back and take a look at the other clones to see if the numbers end up being the same. We did a sketch analysis on a lot of data that was done back in the 1980's on those products. For the most part, we just used that for cell enumeration, but they've been able to quantitate the level of binding that can happen on those cells.

DR. VOGT: And I've used -- The Cytotrol is a great product when we were doing the genotyping and phenotyping. We did CD4 quantitations based on those same methods and they're higher. The CD4 staining is a little stronger. In our hands, it came out between 70,000 and 80,000. It was very consistent. I don't know why I forgot about it. We used it for years.

DR. MARTI: Last comment.

DR. SHAPIRO: Another point, I don't think anybody has really talked about is indirect immunofluorescence and the system that Philippe Poncelet worked out the Quifikit system has been looked at. I'm quoting from basically what the literature says is that Quifikit tracks really well with quantiBRITE. For an ad hoc, but when you start moving something and you take a first shot at quantification, it may be advantageous to use the indirect methodology if you could standardize it because then you only use your one developing antibody to quantify a large number of different antigens.

DR. FISCHER: This is tough to do with a multi-colored.

DR. SHAPIRO: What?

DR. FISCHER: It's tough to do with a multi-colored system.

DR. SHAPIRO: I know it's tough to do with a multi-colored system. That is a problem, but the other thing to point out is that when we talk about numbers of antigens per cell being stable, they're not necessarily stable for all different methods of fixation and lyse. So if it really important to make a quantitative measurement, then we may have to adopt practices that are not as well regarded, in the era of HIV or other transmissive virus infections.

DR. VOGT: Dangerous. And the data that Howard is alluding to with Philippe Poncelet - and just reminding you that this is all rehashing stuff - he used CD5 expressing cell lines, various degree heat controlled expression of CD5. So he used biologic calibrators and then everything, as Howard said, was done on an indirect basis so that the indicator antibody was the same and all those things could be translated over. And although it's an unwieldy system and may not be -

DR. HULTIN: I didn't use the Quifikit but in the beginning for CD4 and 38 I tried to do indirect. I wasn't good enough to get longitudinal

standard and longitudinal stability. I just was not good enough. I could do it for a one-shot assay and then everything is good.

DR. VOGT: See, if you'd just published and quit you'd be in business.

DR. LENKEI: They give you the same second conjugate with the same reagent to get the same results. In the immunized case maybe washings, and it's much more difficult to standardize because in quantitation each manipulation you do it's introducing another factor and it's very important.

DR. BRAYLAN: I think we paid a lot of attention to reagents and beads but I think we forget about the preparation steps.

DR. PURVIS: Well, that's coming.

DR. MARTI: I'm going to suggest that it's about 12:10 p.m., 12:15 p.m. We're an informal enough group that we can probably be lax about when we break and stop but I'm going to suggest that we take a break for what's nominally called lunch, telephone calls and checking back with your offices and labs, and then perhaps meet back here at 1:10 p.m. Would that be a reasonable, responsible thing to do? Okay. Thanks. Off the

record.

(Whereupon at 12:14 p.m. a luncheon recess was taken.)

A-F-T-E-R-N-O-O-N S-E-S-S-I-O-N

1:15 p.m.

DR. STETLER-STEVENS: On the record.

DR. VOGT: I guess we've largely reconvened. So before Gerry takes action, I finally found this slide. I'd forgotten that Philippe Poncelet was the first one and I think this was published in 1985, I forget, but it was a long time ago. So he came up with this number of 50,000. Then in work that we did in 1991 that was published in an obscure chapter of an obscure series that has since died I think that Gerry and Abe and I published. And we did this with the Coulter CD4 FITC. This was the lyophilized powder stuff and we ultracentrifuged it because we did have to get red, it had some aggregates and so on. But if you prepped it nice it was a beautiful antibody and cheap, really cheap.

DR. LENKEI: A very good paper. I recommend well coming to --

DR. VOGT: I still that that's true. We did this isoperimetric titration and this two dimensional titration, which you get all these numbers and you get orders of magnitude that are Millions off and you have no idea of what the

answer is going to be. When we got the answer, I didn't know about this. This was considered low. People were saying that the CD4 was 60,000 or 80,000 or 100,000. So we got an answer of 48,000.

Then I found out that there was an Italian group that reported an answer of 40,000 to 46,000. This was invoked by the folks that were doing that trax ELISA assay for substitute CD4 counting. Then Ken Davis and his colleagues at BD did this beautiful study published in that special issue and they showed, using three different approaches I believe, including ansomatic completely non-fluorescent approach.

If you use their intact antibody, their range in their three methods was 46,000 to 50,000. So I thought that made our 48,000 look pretty good. If they used FAB fragments, as if it had been phoned in by God. It was clearly by then a binding of the intact antibody. So you can actually see in the paper where CD4 apparently tends to form dimmers on the cell surface. So that's not such a surprise now either I guess. In any case I think CD4 is probably our best example of quantitation period.

DR. BRAYLAN: May I ask you? The

conditions on how this specimen is formed. If the concentration of the antibody would make any difference. Things like that I don't see what you can make that here.

DR. VOGT: I can only tell you what I know about ours. I believe the methods are described in detail including the prep methods in Ken's paper but I don't recall them. I've not read this paper.

DR. BRAYLAN: I know, but I don't see in those papers is what if you change those.

DR. VOGT: If you change them. I would say all bets are off. I can tell you that the answer that we got here was actually using the immunolyse stain-to-fix technique more or less off the shelf. We also did some buffy coat cells where we stained with CD4, didn't do any fixing and gated on fluorescence so it didn't do any lysing and all that stuff, and came out with about the same number. So at least for CD4 the old Coulter immunolyse procedure seemed to preserve the staining qualities of viable CD4 cells very well through the fixation process and so on. I gather that varies from prep method to method and marker to marker.

DR. LENKEI: Did you indicate the methodology for the other situation on --

DR. BRAYLAN: What would be the best, fixed material, cold, azide to prevent -- I mean, all those conditions are going to have to be considered.

DR. VOGT: I think that's going to have to be case by case. I think that you all sometimes get fine needle aspirates and hunks of tissue and so on.

DR. BRAYLAN: If I have a chance I'll tell you what we have seen with CD20 which is not the same but I'm curious with the enormous differences in binding if you change the temperature or the time of incubation. So I'm perplexed with that one. I don't know if there is a standard of biological, perfect condition to test against.

DR. VOGT: Gerry in our group co-published some things on CD20 binding some time ago. My recollection was when we tried to find a plateau endpoint for CD20 binding on cells which is obviously CD4. The thing just goes up and bang. CD20 never stops, that was our impression. The more CD20 you add the brighter you get.

DR. LENKEI: Now because it was -- you could probably conjugate. I think we can consider that it is a plateau when the difference between two titres is less than 10 percent.

DR. MARTI: Another way to determine, even if it's not at saturation if the negative cells start to move, then you might as well quit or change clones.

DR. LENKEI: Yes.

DR. VOGT: Even though if you do the kinds of subtraction, it appears to be trivial. There is some discontinuity there because there is not enough fluorescence accountable. It's just a failure of calibration down there probably. But yes. I know on the CD4 PEs that we exchanged three or four years ago that Gerry was -- on those we did do plateau endpoint titres and we found beautiful titration, flat endpoints. It isn't just the phycoerythrin. There are some conjugates of phycoerythrin that we found that plateau out beautifully and there are others, the CD20 in particular, just keeps going up and up and up.

DR. LENKEI: I think theoretically we can have some principles, to be on the safe side, and then we can start with that. We can talk about

that.

DR. BRAYLAN: If we are not reaching it right now, why are we doing it? If we are not saturating it, why is that?

DR. VOGT: That's a good question, Raul. I have to sit down, I know. But the question of what is an ABC. Is it a capacity where we say saturation or -- The real measurement we think, we hope, is how many antibodies are bound on that cell. So when we say capacity, we're making an extension. We're presuming we're at or near saturation. We don't know that.

DR. LENKEI: So is that the reason why in this position which I know many of my colleagues are. They want to have the exact number of molecules.

DR. VOGT: Of receptors.

DR. LENKEI: Of receptors.

DR. VOGT: They don't want to know how many antibodies are in there.

DR. LENKEI: We will never have the exact numbers. For example I think if we count the cells in case it's not blood and we know exactly, to be exact we would have to know it's a very quick test how many of these cells there are there.

Because what we titrate, we have to know exactly what we used in this case. The other would be for example to stain on ice. Because if you stain at room temperature, then the time in which the stain takes is different. And so on and so on. There are different moments.

DR. VOGT: I'll certainly be quiet from now on.

DR. SHAPIRO: Presumably you start to get into steric hindrance problems. You really have to have Millions of molecules per cell to do that. So how many molecules of CD20? What's the largest number of CD20 that you detect?

DR. SCHWARTZ: There's not steric between the molecules but there's maybe steric that possibly -- in the epitope.

DR. LENKEI: It's not a problem of -

DR. SHAPIRO: But then if you never saturate, then what does that mean? Does that mean that there are more epitopes than you can see?

DR. MARTI: I think there is a difference between if it's a nice sharp and comes up in plateaus or it looks up and then starts a new binding thing that takes two weeks or two months to finish.

DR. SHAPIRO: The patient's dead.

DR. MARTI: That's another problem.

DR. LENKEI: You have to have enough end product because in this condition or in the companies that are diluting the antibodies, then you have to know that you have enough antibody for the sample.

DR. VOGT: If you really get down to it, nobody ever measures binding capacity by saturating. If you do real binding capacity measurement, you do some kind of scatter type analysis and you extrapolate to binding capacity because you can never saturate. In a practical sense if anyone did steroid receptors which I did years ago, you don't even pretend like you can try to guess at that.

DR. SHAPIRO: But that depends on the test.

DR. VOGT: Right.

DR. SHAPIRO: Right now, some of these antibodies have been engineered in yeast. You have thermomolar binding now. This is for monoclonal, an SCFV fragment. So presumably you could -- Nobody is going to solve it commercially, but if you are playing this game you could acquire

reagents that would let you investigate this.

DR. MARTI: I was just also thinking that, and don't anybody answer, but the CD20 problem as I recall had two other parts to it or perhaps three. You didn't get the same answer if you did it in whole blood versus nitrate glycol or versus purified B-cells. But don't anybody answer because I don't know what it means.

I'm trying to get back on schedule, not that we're necessarily off. The next person who is going to talk to us is Larry Lamb. Larry has been looking at CD69 under a broader range of conditions trying to do it quantitatively. Let's see what he has to say.

DR. LAMB: I come at this from a little bit different angle because my training is transplant immunology. The reason I come to a flow cytometry meeting is that I inherited a diagnostic laboratory when I finished my fellowship, and do some of that as well. We undertook this a few years ago to take an old test and try to make it a little better so that it showed some real-time changes and that sort of thing.

CD69 based immune function assays and we use the quantiBRITE kit gives you a fast

turnaround and an approximation of some real time changes. If you are doing a mitogen assay with thymidine incorporation, you're looking at what the immune system was doing five or six days ago. You're not looking at what the immune system is doing now. Also you have the ability to subset cells with flow so that you can see what's activated and what's not activated.

In a thymidine incorporation assay, you just have this soup of cells floating around in PHA or Con-A or whatever and they give a number. That is some cell might have taken up some amount of thymidine and showing something about proliferation, but you don't know how many are in there that are dead either. So it's an imprecise assay.

So you can do this. You can combine surface phenotypes, surface activation markers, proliferation markers like PRDU and intracellular cytokines. We thought it might be fun to see if we could do this a better way.

This is a prime example of why we did what we did. This is 25 patients that recovered from a bone marrow transplantation without overwhelming infection, without clinical grade

graft versus host disease, and without early relapse. In other words, these are normally recovering patients. They also all receive the same type of transplant. They received a haplo disparate bone marrow transplant. It's a close knit group here.

As we can see at 30 years down the road, we have standard error bars this wide on pokeweed that overshoots the percent response. Con A's down around normal. PHA is still depressed. So the question is what exactly is this telling you. Interestingly enough Medicaid pays for this. They don't pay for the flow but they pay for this. I still can't figure that out.

Quantitative flow cytometry determines the number of antibody binding cells. You all know that. It's less subjective in the determination of percent positive and ideally of course requires all the things that we talked about today, calibration software, tightly controlled concentration, fluorochrome on Bs and one-to-one unimolar conjugates. I learned that word today, Bob. Thank you.

Quantitative determination of whole blood lymphocyte activation and functions is what

we want to use 69 for. It's also been used in HIV labs as well. It is a good indicator of monitoring immune function recovery post transplant in addition to immunophenotypic recovery and also can be useful in Type I diabetes and lupus. It gives you an index to immune function.

One of the things that a mitogen assay does not tell you, and we found this time and time again, is that if a patient is recovering from bone marrow transplantation and their immune system is already activated, the mitogen assay is useless because it will tell you that you can't activate the immune system because you are getting very little proliferation. It doesn't tell you that the cells are so far out there they are not going to proliferate no matter how much mitogen you put on them. But CD69 will tell you this. It will fluoresce high if the cells are already active and you'll see the little change. So it can be used to look at baseline states or even in autoimmune disease to compare with what we consider a normal range.

Methods was very simple. We took whole blood in a sodium heparin vacutainer. By the way we did find that there was a significant time

problem with the sample if it was kept over four hours at room temperature. We could ice the sample and it would be fine for 24 hours. But if we let it slip beyond four hours we were starting to see rapid degradation of the CD69 on the cell surface. A lot of this work was done by my student who is now a first year medical resident, Brad Lindsey, who won the little prize last year.

DR. STETLER-STEVENS: Young Investigator.

DR. LAMB: The Young Investigator Award at the CCS meeting. Stimulating the cells according to manufacturer's instructions, PHA 20 micrograms per ml, four hours, 37 degrees Centigrade, labeled cells, analyzed with the flow cytometer and took our standard curve and plotted the number that came out, the geometric mean against that.

What we saw, resting is pretty low of what you would expect that's on normal individuals. I think I said the people in my lab are normal and some wouldn't. This is CD28, staphylococcal enterotoxin B in combination there and PHA. For both subsets, this is CD8 plus CD4 equals CD3 basically. It's sort of in the middle there

averaging out the two.

DR. VOGT: How long after stimulation?

DR. LAMB: We stimulated for four hours and then we ran immediately. On bone marrow transplant patients, this is 100 days following bone marrow transplant. Resting is actually a little bit higher. Flipped between the two, one of these days I have to put both of these on one slide. It's a little bit more because the immune system is a little more activated. That's the actual real deal there with SEBC 28 but I know that it has to be wrong. There's an outlier in there. But the PHA was a little more reasonable. If you look at the PHA they're about half at 100 days what the normal was. You're still immunosuppressed at that point functionally.

I'm going to throw in another test here just for your comparison because I've been harping all day long about why isn't this used in more laboratories and I'm going to answer the question that I asked you because I think if I can ask it of you I have to be fair and answer it as well. Why isn't everybody doing this if it's so good? Because everybody in bone marrow transplantation or immunotherapy is looking for a way to measure

immune function reliably.

People want plug and play. We are fixing to undertake a study looking at using quantitative flow cytometry and this little kit I'm fixing to talk about comparing the two in the immune function of lung cancer patients. There is some data out there showing that response to chemotherapy in stage IV lung cancer may be correlated somewhat with the function of CD4 T-cells.

So we're going to use a couple of methods. This particular method is a bio-luminescent assay. It's a clinical correlate of cell immunity. It is somewhat cytometric. It fits ISAC's definition anyway. It can do it in 24 hours or less. It's non radioactive and can use whole blood or PMBCs, cost effective and can test with multiple antigens.

Using this method which is manufactured by a company called Cylabs in Baltimore, lymphocytes are stimulated with PHA. They are incubated up to four hours to overnight. CD4 cells are magnetically separated and washed. Lyse to release ATP and light intensity is measured on a luminometer. Looking at this reaction ATP plus

luciferin using luciferase as the enzyme breaks down to oxyluciferin and light. The light is right at 562 nanometers. They're internal work so the intensity of the light emitted is directly proportional to the amount of ATP in this sample. They have a standard calibration kit as well that comes with it that gives you the same type of line as you get with the CD69 kit.

You look at the comparison with lymphocyte proliferation assay looking at DPM on the right and ATP on the left, the two curves tend to track together fairly well. That's that answer. That's what we are trying to get here. Gerry is going to talk a little bit about some of the things that we found when we did some comparisons using the CD69 assay between a group in London at Royal Free where I did my sabbatical in 2000 and here at my laboratory using the same lot of CD69 and the same lot of Calibrite beads and the same lot of quantiBRITE beads as well and see how that came together.

I'll stop here. I'm just here to primarily paint the background for Gerry to talk about some of the more technical issues in the study that we've been doing together over the past

three years.

DR. MARTI: Are you going to abandon doing the flow based with CD69?

DR. LAMB: No, I'm not. I'm going to compare the two with this lung cancer assay and see what the two look like. There are problems with both assays. The reason I'm looking at this is this is a plug and play system. This is something that you can hand a bench tech and say put solution A into solution B, cook for four hours, toss lightly and serve, and it's done. Flows are going to be a bit more complex. I think It's going to be interesting to see if there's anything that we lose with the more simplistic assay that we don't with the CD69 assay.

DR. DAVIS: With your CD69 stuff, why even make it quantitative? What's different about that as opposed to just percent positive for 69?

DR. LAMB: Because like CD38, it presents as a smear. It does not present as a --

DR. DAVIS: But is there superiority of one data over the other in your patient population? I would think if you looked at your bone marrow transplantations you would see a rise of percent positive as well.

DR. LAMB: It becomes subjective as to where you're going to place a marker to determine percent positive when you have sort of a cigar-shaped population going across quadrant 1 and 2. The tech or the operator or the analyzer or the head of the lab can all disagree about where the quadrant 1 and 2 are going to be separated if you are looking at a two-dimensional dot plot. So to get a geometric mean of the population takes some of the subjectivity out of the discussion.

DR. DAVIS: -- that's the question.

DR. LAMB: Then you are left with this is activated. This is really activated. This is kind of activated. I don't know whether this is activated or not. That's different things to different people. I see your point. Sure you can tell the difference right away when you are looking at it, as whether -- how hot the cells are but each further number might be better.

DR. LENKEI: You can get significant differences in the intensity without having differences in percentages. This is very important when you are using this method.

DR. DAVIS: And the opposite is true.

DR. LENKEI: You can get the cutoff

which can be quite good.

DR. DAVIS: I'm just asking why you went that direction.

DR. LAMB: That was mine because we just didn't want to leave it to subjectivity.

DR. VOGT: Could we ask Lance that same question? I mean, I think when you started doing this, you were looking at placing a cursor somewhere and saying if CD38 percentage went up.

DR. HULTIN: I'm not a statistician but the univariant chi-square for predictive value was definitely higher in the fluorescence intensity.

DR. VOGT: So you did try dichotomizing and found it to be less.

DR. HULTIN: Sure, but there was another issue too that quantiBRITE helps with now and I've shown it with Jeffrey Wong's sphere techniques. Sure, I can keep a fixed cursor setting, but now I know if you want it to be an assay that has cross-lab comparability, it is harder with the smear to generate similar percent positives than it is with the quantiBRITE method.

However in more recent cross-lab studies, I've used the approach of okay, I've characterized the instrument objectively and I tell

them where to put the cursor. You could do that with the quantiBRITE as your threshold percent positive. It is a very good approach. I've shown that it really narrows the variation across labs. If you just let them use an isotope control and count their own percent positive, the variation was much wider.

DR. LAMB: Bruce, can I show three more slides? It might help us arrive at this decision.

DR. FISCHER: I have another question to ask you because you mentioned at the very beginning about the drop in CD69 when you leave the two at room temperature. I'm sure many people in here have had probably similar experiences. Is that only in sodium heparin? How about sodium citrate or EDTA?

DR. LAMB: You have to use sodium heparin.

DR. FISCHER: That's for both the flow and this?

DR. LAMB: I think so. I'm not willing to preach gospel on this one because I just got the kit, but I think it's probably so.

DR. FISCHER: But you noticed that with the flow ones that 69 went down when you used other

than heparin for the blood?

DR. LAMB: Yes. Bruce, I want to share just a couple of slides here on OX40. Then I'll let you tell me as a pathologist what you would like to see. We did a study with Becton Dickinson to look at CD134 expression in graft versus host disease. We published this in "Cytometry" about two years ago.

What we thought at first was we might be able to have a predictor of graft versus host disease. That didn't turn out to be the case. I'll show you what it did. This is 28 patients looking at percent positive. Look how tight those are, recovering from bone marrow transplant. In normal CD134, CD4 cells is right down around here at eight percent.

Of note, none of these patients experienced clinical graft versus host disease. They didn't have a rash but they did have what we would call an activation immune system basically because of the allogeneic transplant. This came up to about 40 percent positive and stayed there up to about 135 days when we stopped doing the measurement.

But what we did see is when we looked

at patients who developed graft versus host disease, we saw in patients that responded to treatment a spike here and then a gradual decrease when treated with high dose Methylprednisolone. This is around 60. The other was around 40. This is around 60 as well. These are two separate patients. You see the spike, you treat and then the next couple of days you start to see OX40 expression come down.

The patients who didn't respond such as this one for instance got Methylpred. He's around 75. It came down a little bit. It didn't really respond. At ATG here, everybody will know why we can't measure anything here because you wiped out your T-cells. He comes roaring back, is treated with OKT3 again, drops off and then ultimately levels off. This patient survived.

This one had two doses of high dose steroids and ATG and only experienced two or three days when he was down and came back up. At this point, the patient died of graft versus host disease. As a pathologist would you like to have a number for that to work with? An ABC? Or would something like this be sufficient to tell our physician he's got a rash now but five days from

now if he's still up this high don't treat him with methylpred but treat him with more definitive?

DR. DAVIS: You have to compare both ways unless it's sensitivity and specificity. Whether you call it ABCs or Zebras doesn't matter. What matters is the clinical correlation with the parameter you are going to report out.

DR. LAMB: That's going to be a little uncomfortable making a treatment judgment in a situation for all I can see with my eyes is grade one to two graft versus host disease and trying to correlate back with a number to decide whether I'm going to give this person heavy therapy or not.

DR. BRAYLAN: Isn't this what we do every day? We look for the scope but we have some subjective impressions on what we see. One is positive and one is negative. Big decisions are made on those subjective impressions but actually they're probably good because we can test everything for numbers. Then on the statistic side, that's not going to be clinically --

DR. FISCHER: There are going to be some diseases where you are going to need a number because you're not going to be looking through a microscope and seeing it as a vision.

DR. BRAYLAN: I understand. I'm not saying that is the way to go but that's the way it's done.

DR. FISCHER: Since when you're going to look through a microscope and you're going to say histologically yes I know technically what's wrong with this person but there are going to be other diseases that you can't do that because they don't present as a case that you can look through a microscope. That's where you need this.

DR. VOGT: Larry, we're still talking about comparing two different sets of numbers. What Bruce was saying is the bottom line. You can get numbers from that so you do your raw curves and you see whether a quantifiable expression parameter gives you a better predictivity. I don't know if that's ever been done really now that I stop and think about except in Lance's case.

I do remember now that Janice presented I think in Canada at Frank's meeting the fact that the chi square of the significance of a percentage above a cutoff point that correlation with clinical outcome was not as strong as the quantified CD69 expression as an ABC. That's the one case I know of. I had forgotten about it but I do now recall

that data.

DR. LAMB: To give you an idea -- and this is a parody on what Abe said about reinventing the wheel but to give you an idea how far this technology has come, this same study almost word for word after we published this in 1989 was published again in Blood late 2002. It was the same measurements. We're still reinventing the wheel here looking for a way.

DR. STETLER-STEVENS: You are looking at a subtle change in the percent positive cells. Thinking about somebody setting their quadrants one day and then another day and then a week later they are setting their quadrants without good standardization. You could get changes. You could look like you are continuing to have decrease or you could have level number of percent depending on how good the person is, how standardized you are setting your quadrants. Looking at an isotype control, how are you going to do it?

DR. BRAYLAN: Our focus, the statisticians' focus -- you're not supposed to reach percentages out of the degree. That's not efficient. We're creating two calculations when you need only one.

DR. MUIRHEAD: You can do it but it's probably not --

DR. STETLER-STEVENS: So that's why I wanted this clarification of antigen expression rather than to take big look at the "hot dog" as Carlton calls it and slice it someplace based on some arbitrary rules.

DR. BRAYLAN: But even if you had rules, it's unclear. You've got conjugating positive signs of the population or negative signs of another population but the two populations are not separate.

DR. STETLER-STEVENS: Exactly.

DR. D'HAUTCOURT: In a sense, if you want to quantitate something you must recognize that we'll say if you have a speed for distribution you can make a good quantitation because the threshold in place is always an estimation but not at all.

DR. STETLER-STEVENS: I would think that for a unimolar distribution the quantitation would be better than a so-called positive because what does that mean when someone says present positive for X. I say they all are. They say how much above the isotype control? Eighty percent.

So it's 80 percent positive. No, they are all positive but it's dim. To me I would rather say it's a dim positive which is an off the cuff quantitation.

DR. VOGT: I think that's what Jean-Luc was saying is that if you don't see a dichotomy then you can't identify a population or claim that there's something positive versus negative. Is that it?

DR. D'HAUTCOURT: If you don't equalize the two populations you can not give an exact estimation of the FITC.

DR. HOFFMAN: But if you are quantitating inaccurate and you are quantitating the important one, you can say 10 percent or above this level of MESF or antigen binding or whatever.

DR. BRAYLAN: You can express that as positive or negative.

DR. LENKEI: But you see the significant differences nevertheless and you have clean correlations looking at many of these for the whole population because it's such a large distribution from negative to positive. But nevertheless you can note the significance.

DR. MARTI: Dr. Hsi has had some

experience with CD38 on CLL lymphocytes with a quantitative flow. Dr. Hsi, would you like to comment on this?

DR. HSI: I can say just this thing about percentages and ABC. At least the data we had on the SLOT study was that quantitation gave you a better clinical predictor of progression compared to a percent cutoff. So then we looked at also the patients that were discrepant. In other words, the ones that you would have thought would be "positive" for CD38 -- I'm sorry, the ones you would have thought would have been negative for CD38 by calling them based on percent but antigen quantitation fell above that. Those actually did worse and so it was a better way. Those discrepant ones really behaved more like what you thought according to antibodies found per cell number versus the eight percent. I'm sure there will be examples where a percent positive or negative is not as good as a quantitative number. I'm sure the same thing is true depending on the system that the user has.

DR. LAMB: To sum up for me, for this type of quantitation and function, a couple of the practical values, comparison of recovering T-cell

activation with normals, to be able to look a baseline lymphocyte activation statement you can't do it with the current state-of-the-art. To look at recovery of lymphocyte function in addition to numbers so that it gives you a clearer picture of the immune system, what's the state of each predictor cell, and also look at immunodeficiency disorders. A big thing that's come up now with renewed interest in smallpox vaccinations is that the market company that I'm working with is in discussion with Tom Ridge's group about perhaps taking people who are shy about getting a smallpox vaccination because they are concerned about their immune status and just testing it.

DR. VOGT: What about their hearts?

DR. LAMB: That's a different matter. You think about something that could make somebody rich overnight. Something like this could. Then all three flow manufacturers if this would be done by flow it would be jumping in this tomorrow morning in their board rooms. That's something that could happen. Also monitoring cellular therapy protocols.

Right now, we can infuse a cellular product into a patient, go through all the process

of taking a T-lymphocyte, incubating it on a dendritic cell, then expressing whatever antigen you might want, expanding it up and shooting it into the patient and then just praying that something is going to happen because you have no idea what that cell is doing. But if you can select this with your tetramer and then by flow analyze it with CD8 tetramer CD69 two or three weeks down the road, you not only know that your cell is still circulating but you know if it's still activated. I'll stop with that.

DR. MARTI: The whole area of trying to find markers to predict severe graft versus host disease is really an interesting area. Dan Fowler at our institution is looking at intercytoplasmic markers. At the risk of misquoting him, I believe that intercytoplasmic gamma interferon in monocytes appears to correlate with whether or not someone is going to develop severe GI graft versus host disease. It seems to me I had another comment I wanted to make. Bob, can you put those few slides that I have on?

DR. VOGT: Which ones?

DR. MARTI: I don't remember what I called it. If you didn't save it. I put Larry's

name on it. I can give an introduction while he's looking for it.

DR. VOGT: I remember. Just one second.

DR. MARTI: When Larry was on his sabbatical and came back to his own lab, apparently both labs were doing CD69 activation studies. As Larry said, they were using the same antibody and the same calibration curve. I saw this as an opportunity to do an interlaboratory comparison that was supposedly using the QuantiBRITE system. It looks like one is missing there but that's okay.

In the lab in the U.K. essentially they looked at CD3 and CD4 lymphocytes and also CD3 and CD4 looking at CD69. I'm trying to figure out -- I made these myself so I should know what they are. Anyway, it was the population of CD3 and CD4 and CD8. CD69 was looked at each of those. Then we made single parameter overlays showing the difference between in this case on CD8, the expression of the CD69.

On the next slide, basically these were the calibration curves that were carried out over several weeks in the U.K. lab. They were quite happy that by and large they tended to overlay one

another. Using these calibration curves, this was the range of expression going from baseline to the stimulation for CD69 on CD4 cells. This was the expression for CD69 on CD8 cells.

On the next slide, it's the calibration curves being compared. Actually, in Larry's lab they looked at both a set of calibration curves for both the patient population that they looked at and also for normal controls. We were satisfied that those looked pretty good.

On the next slide, this was the study that he did in his lab looking at CD69 at rest using three different Con-A, PHA and Staph. What is this one, Larry?

DR. LAMB: Staphylococcus.

DR. MARTI: Again looking at 69 on 4 and 8 and doing overlays actually there are both conditions here for PHA and SEP. On the next slide, it is the comparison of CD69 expression on 4 and 8 for PHA and for 4 and 8 CD69 with stimulation for SEP.

The next slide should be the values that were obtained for the resting stimulated PHA. Resting stimulated CD8 were compared to the values found in the U.S. lab which are across here. The

differences between the two labs are summarized here. Here the differences are in a negative direction and the other three, the U.S. lab found it at a much higher level of stimulation.

One wanted to know why. If you go into the literature and look at the papers where this has been done, there's a variation in the literature that far exceeds these numbers. So I wasn't too concerned about that. On the next slide is the comparison of the calibration curves supposedly using the same CD69 antibody, the same lot of CaliBRITE beads. I can't say for sure about the PHA and Con-A and SEP. Certainly the normal subjects weren't the same.

I thought that in trying to explain the differences between the two labs, the most likely place to start was with the calibration curves. I also was naive enough to think that a group such as this would be the place to bring and talk about what causes the differences. Why didn't these two labs get the same set of calibration curves? They are very reproducible within their own lab but they are not very reproducible between one another. Why?

DR. HULTIN: Do I hear you saying then

QuantiBRITE beads were all the same?

DR. MARTI: That's correct.

DR. HULTINN: There's a different PMT setting.

DR. SCHWARTZ: What's the instrument set at?

DR. HULTIN: Now because with the calibration.

DR. MARTI: As far as the calibration you are right. I shared that with the change in PMT but just in the position of the calibration.

DR. HULTIN: They don't have the same analysis.

(Discussion.)

DR. MARTI: What do we call all these things that you are all saying? What do we call those?

DR. SCHWARTZ: Instruments.

DR. MARTI: We call it a unified set-up, right? Everybody that goes in and turns on an instrument and goes to get a cup of coffee comes back and starts. I remember years ago we used to find it very useful to just sit down and calmly talk about what you do. Not everybody sets their instrument up the same. What are the two major

differences in setting up an instrument worldwide? On this side of the room, what do you use to set up your instruments? One side is going to set up with positives, right? Something bright. And the other side is going to say no, I set up on negatives, right?

DR. FISCHER: How do you define center, Gerry? It's a broad number of things that we do to an instrument if we are going to have it ready to use for analysis. You set the PMTs number one.

DR. MARTI: How do you set the PMTs?

DR. FISCHER: I use the negative population and a positive control.

DR. MARTI: That's a very dangerous thing to do on a non-digital signal processing system because of the way the instruments work something that's negative four orders of magnitude in the negative direction or something that's three or two or one all get put in the first channel. You don't know where your negatives are in fluorescent space.

DR. FISCHER: In relation to the positive that's what I said. It's all one sample. We traditionally choose molecules for our positive controls that have both a positive and negative

population in them clearly separatable so that we can set the negatives at a certain position every time we run that test.

DR. LENKEI: What is your positive?

DR. FISCHER: It depends on the sample you are using. We use anything from CD4 to CD8, sometimes 19 depending upon the T-cell.

DR. LENKEI: That is not the same cell you are using.

DR. FISCHER: Hell no, I work in clinical specimens. Nothing is the same.

DR. LENKEI: Exactly, so you put your positive on a number of an intensity which by principle is variate, because if I have a patient with BCNA I would have one CD19, and if I'm normal control I would have another CD19.

DR. FISCHER: This is post -- we're talking about post set-up. You're talking about -- because we always QC all those for B. That's a standard use.

DR. SCHWARTZ: Set up or validate?

DR. LENKEI: Yes.

DR. FISCHER: Validate.

DR. MARTI: The single strongest criticism against setting up on negatives is that

you're setting up on noise or close to noise. One of the best things you'd like to know about your system as scientists is you would love to be able to say what is the noise in my system. If you know what the noise is you won't make measurements below that. But if you set up on negatives you will never know. You will never be able to measure that.

There may be other reasons why these calibration curves aren't over. I think that the fact that there wasn't a unified set-up that implies that the window of analysis which has been mentioned several times. You know the scale that we use for measurement in fluorescence goes across several of these rooms, right? It's positive infinity on this side and negative on the other. So where you are on that scale at least in olden days was you were able to look at four logs somewhere in there.

Now today they tell us the dynamic range is such that we can look at I'm afraid to say 12 logs because they used to laugh at me when I said five logs in log log space because that didn't make sense in engineering. I guess the windier way to say it is the linear dynamic range is now 12

logs. How do you express the linear dynamic range with digital signal processing?

PARTICIPANT: With ours, we have 18 disks.

DR. SCHWARTZ: 200,000 channels, it's nothing.

DR. MARTI: So stay with the old channel analysis.

DR. FISCHER: That's linear in that range.

DR. MARTI: That is the linear channel. Basically the thought I was trying to get out is that this window of analysis sets where you are going to be looking in fluorescent space. You usually have to be careful at the ends of that just because of the way the instrument functions but perhaps where you are in that space.

DR. WOOD: You're showing these curves here and this is really a log off plot. Aren't you just looking at the difference in gain? That's really it?

DR. SCHWARTZ: Yes.

DR. WOOD: That's all that it indicates. The gains are different because it's clearly two systems.

DR. D'HAUTCOURT: Jim, I have difficulties to understand why if you are using ML positive light you get so diverse results because the calibration is there to solve this problem. If you use it correctly, let me say in the lab in U.K. and in the lab in the U.S. in this they used the four beads. You use only in this case the three, the second one and the fourth and you consider the third bead as the test. If you check you see that they get the same results. If they don't find the same result with patients then we would say that there is another problem. But it's not a problem of the calibration.

DR. WOOD: All that this is doing is bring back the dangerousness. If you had done this in linear domain all that you would have done would see that the line is shifted up a little bit. The slope would be the same.

DR. SCHWARTZ: Not in linear domain.

(Inaudible.)

DR. MARTI: When you are looking here you can't see a difference between an antibody combining capacity or AB/C I believe is the BD nomenclature of 1440 versus 10,889. That's not related to the extrapolation off of those two

different calibration curves. It's due to something else.

DR. WOOD: For example, if you were to use the fact that the negatives have to be in a certain position, you would have to be running at a higher gain than if you were using positive systems. Therefore you would be operating at two different gains.

DR. MARTI: But I thought in log log space you don't talk about gain anymore.

DR. WOOD: No it's Y equals ABX. So actually any function is straight in log log space. Those could be parabolic functions for all we know in log. The slope is related to the power. The offset is related to the gain that's before it. So if you operate at two different gains, you set your PMTs at two different voltage levels, then you're going to have two separate curves. It won't be the same.

DR. SCHWARTZ: The sample will read the same on both slides.

DR. MARTI: My naive approach to this was that it was part of the behind the scenes things that you should be able to bury things like filtration, filters.

DR. WOOD: That's part of the problem. This is an issue of display. When you are dealing with a log log plot and when you start thinking of it in a linear pipeline set, you're deceived real fast because it looks like it's a linear relationship but it's not. Those two could be parabolic, cubic, quartered.

DR. MARTI: So what you're saying is that if I slide one set of those calibration curves one way or the other I can do that legitimately. Then their answers would be the same or much closer.

DR. WOOD: It's certainly -- all it is --

DR. HULTIN: This is a question to you. Is the top bead too close to the end of the log end? Would that affect it?

DR. WOOD: No. You have to look at underlying relations that you are dealing with.

DR. HOFFMAN: Is this to clarify that the vertical axis is being channeled? It's not a quantitative measurement.

DR. D'HAUTCOURT: It's a different technique.

DR. SCHWARTZ: I've run a study like

this with a unified setup and a non-unified setup. Theoretically the calibration curve should correct for any set-up situation.

DR. D'HAUTCOURT: Yes. But if you remember --

DR. SCHWARTZ: In practicality you get a little bit better correlation when you have a unified set-up, about five percent more. It helps in a practical sense a little bit but theoretically you could set it up anywhere and as long as you're using a calibration curve properly you'll get the same answer. That's what calibration setups are for.

DR. D'HAUTCOURT: Yes, but to get a big difference might be something we've seen before but we'll say that it's something else.

DR. SCHWARTZ: Yes, if you get a big difference then something else is wrong.

DR. HOFFMAN: But that's not a big difference necessarily. It's not a big difference in gain.

DR. SCHWARTZ: When you look at these numbers that come off the calibration curve, those are a big difference.

DR. HOFFMAN: But the differences are

here.

DR. SCHWARTZ: Those are actually ABCs. So that's a real serious problem.

DR. MARTI: This is off of the BD program. The BD program was used to generate these numbers. In fact I wasn't going to go into this but because the data was collected in two different places and was being analyzed in a third lab, serial numbers and lot numbers, I had to get specific permission to get a subclass piece of software so that we could analyze it. Then of course it all had to be checked. I don't think we have manipulated the data or that we knowingly did.

DR. WOOD: What happens here is when you're dealing with a linear domain, if you just have the equation Y equals MX + B and if you change M then what you'll see is your slope will change. If you change B, you'll have an appropriate change in just position. A negative B will go this way. We're used to looking at that in the linear domain. It's real easy to understand that.

The problem is if you look at this in the log log domain, this type of mindset does not work. In fact in the log log domain people are trying to use a linear mindset to interpret this.

Again if you take this equation, which is what the log log plot works for in a native format, you can see that your slope here is related to the power of the function. As long as you're dealing with a power function, it's going to be straight. There's no way it can't be straight. In fact a trick in dealing with data is that if you don't know anything about your data and you want it straight just plot it along log and it will be straight.

Your offset is related to the gain. That's why in a standard log plot if you increase the gain, everything just moves up and it looks exactly the same. So all you've done is just change the gain. In this over here where you have the two curves, you have the same slope and all it does is move up, all you've done is changed the gain. The functions are the same.

Now we don't have any information here as to whether these are linear functions or cubic or quartered, third power, four power, fifth power. They could be to the 100 power and they would still be straight.

If you take a linear function here where you set E equal to one, what happens then is that you will see out here in the ranges where this

dominates the intercept, you'll see a relatively straight line. Then it will either do this or it will do this based on the value of what C is.

This, if you will, is what you're going to be looking at in the very first decade. Because what you're dealing with down here is the point where your background, which is what this factor is, starts to dominate.

DR. SCHWARTZ: That's another reason why you shouldn't set up on negatives.

DR. WOOD: The problem here though is that what you shouldn't be doing is using this log log presentation. It just doesn't work. In fact it incredibly misrepresents the problem when you are doing things like compensation and so forth.

Do you ever think about this? You are trying to compensate your data. First of all, you start out with something that looks like this and you want to take this and straighten this out. As you're adding compensation, if I complete this, what you're doing is you're taking this and doing this. It's like a steamroller rolling over it. This little flip at the end will always be there. You can't get rid of it.

DR. SCHWARTZ: What happens when you

get all the compensation to extend the other way?

DR. WOOD: No, you can't get rid of it.

DR. SCHWARTZ: Nobody can overcompensate.

DR. WOOD: No, what happens is that this down here as you go out there and start to overcompensate is that you lose any information that's down here. It's just lost. It's thrown below zero. Now if you take a look at this in the linear domain, you'll actually see when you do the compensation the two populations just splay right out, separate out exactly the way you want to see it.

In fact when you take two populations like that that have been perfectly compensated in the linear domain and then look at them in the log log domain, you will see that they actually look like that. They are not compensated. Unfortunately this is not a simple ten minute discussion. This is where you have to really sit down and look at it and touch it and feel it and play with it and see why this is so.

The problem is that we are taking a log log domain with a linear mindset to it. You can't do that. Therefore when you do calibration curves

within a log log plot, you have to set up this windows of analysis. It's a crutch in order to work within the log log domain.

If we were working in the linear domain, we would have never even been there. In fact compensation would have been a non-issue because compensation would have been something that would have been very intuitive. But unfortunately it's become a real problem in the flow world because people are trying to take this curve here and flatten the thing out.

DR. VOGT: Again I certainly agree and you helped me through this last year in San Diego and I have gotten a somewhat increased appreciation but not nearly enough I'm sure. I'm going to ask Dolph, who's here. He and I had this conversation a couple of weeks ago. Our presumptions in quantitative fluorescence are based on the fact that there are so many irregularities that can occur in fluorescence, quenching interfilter effect and so on.

So we have taken the approximation that B equals one. In other words, we're saying that for every increment in fluorochrome mass you get an exactly proportionate increment in fluorescence

intensity reading. That will only be true over a narrow range. Then all of the tenets will fall apart. But within the range that we presume to be working which is from the range of cellular autofluorescence up to a few hundred thousand MESF, not even a Million I would guess, we are presuming that B equals one so we therefore do have a Y equals MX + B situation in that range.

DR. WOOD: I'm not sure I can agree with that because what you have there --

DR. SCHWARTZ: You're dealing with instrument problems.

DR. WOOD: It's just related to interpretation here.

DR. SHAPIRO: It has to do with a comparative scale. You are correct in a sense that if we neglect the energy transfer and the quenching and the interfilter with X and all these things that if you have one fluorescent molecule you will measure a signal with X and if you have two fluorescent molecules you measure a signal with 2X, and that probably holds across the board. But the conversion to a log scale by log amplifiers screws that up somewhat. Even if you go to the digital system, which are pretty much ideal in a

logarithmic conversions, when you are dealing with data on logarithmic scales, the reason that the compensation curves splay out and give you what I would call the curly gates -- if you go up Constitution Avenue you have to pass through the curly gates. The reason this happens is that compensation is a deterministic process but the measurement is a random process. You are subtracting a fixed number from a number that has a great amount of random variation on it. So when you compensate something that's out along the end of one axis there's going to be more noise left than there is when you compensate something that's in the bottom corner that have low values of both axes. You just can't get around that. There is no compensation that will get you into quadrants.

DR. WOOD: In the log log domain.

DR. SHAPIRO: In the log log domain.

DR. D'HAUTCOURT: I cannot explain so many things like you. I read your paper you published two years ago about this and after three readings, I stopped. I cannot understand everything. But if you want to come back to the top, this is for me -- if you construct a curve and you see that this curve is quite good because it's

really linear.

In another, you have the same that will say between this and this you have a linear relation between the intensity and the number of molecules. If you make a measurement you must use this range to make your measurement. If you make your measurement between this and this or between this and this, you must find the same BE molecules. This is the whole of the calibrator. If we do not agree with this, we must stop the discussion because there is no way to make calibration.

DR. SCHWARTZ: Then why are they are getting different answers.

DR. D'HAUTCOURT: This is another problem but what I don't agree with is that it's not a unified window can solve this problem.

DR. MARTI: Go back to the numbers.

DR. SCHWARTZ: It will not. You are absolutely right.

DR. D'HAUTCOURT: Okay.

DR. VOGT: The question is we have what looks to me to be a roughly proportionate bias here of about three and a half fold. In other words, the difference between the 4,000 in the UK and the 12,000 or so in the U.S. and then the difference

between 4,000 in the U.K. and the 4 plus thousand.

DR. SHAPIRO: But we know that from the two curves because the curves are approximately parallel. That should not be a constant factor.

DR. VOGT: It looks like there was a screw-up in the calculations is what I'm trying to say. It looks like somebody used the wrong curve to calculate the results because that's a roughly proportionate bias which I think Howard is right would be about explained by the gap between those two curves which is roughly half a log. That would be about fourfold or so. Threefold. So I think somebody made a mistake here, Gerry. That's what I'm saying.

DR. MARTI: Be careful. You might hurt my feelings.

DR. SCHWARTZ: It was the other guys that did it.

DR. VOGT: It was the French. The French came in overnight and sabotaged the whole damn thing.

DR. MUIRHEAD: I think if I understood what you just said you're comparing top to bottom. I'm looking across and saying what is this telling me about the biology. Item number one up at the

top says you're resting CD34 and CD8s at about the same number of CD69. Item number two says no. There was almost a 60 or 70 percent higher level to start out with on my resting CD8s.

Now look at the stimulation. Got more stimulation and more CD69 up regulation on the CD8s than I did on the CD4s after stimulation. I'm getting a biologically different conclusion out of those two sets of data on something where I supposedly calibrated. I agree with Jean-Luc. It's not the calibration part that's at fault. There is something else different between those two labs.

DR. LAMB: If you take the two calibration curves and remember how Brad did this, he normalized the first B on both, put them together. Those differences dropped to about 1.5 fold.

DR. MUIRHEAD: When you say normalize, what do you mean?

DR. LAMB: Close those sets of curves.

DR. SCHWARTZ: He took the curve and you shift the values from your samples equivalently.

DR. LAMB: Yes, shift the values from

your samples equivalently, you get a lot less play. We believe that that difference by itself can be explained by the fact that we use two different lots of PHA. Different PHA gives different biological response depending on the lot that it's in. But the point is, Bob --

DR. SHAPIRO: Wait a minute. The fact that your two calibration curves are different that's not the major problem that you're dealing with here.

DR. LAMB: Actually what you've done is you've shown that you've had two different lots of PHA. Your calibration curves worked. Your calibration curves corrected for these differences and proved that you for some reason choose to use two different lots of PHA.

DR. FISCHER: And also that the water in the U.S. is different.

DR. LAMB: Or something like that. Or that people in England have less stress than we do.

DR. SCHWARTZ: They don't hold these meetings.

(Discussion.)

DR. STETLER-STEVENS: Let's move on.

DR. MARTI: Anyone who has further

comments about this experiment can talk about it in the hallway.

DR. STETLER-STEVENS: Let them present all the data before we stop and try to figure everything out. At this point let's get the whole picture. Bruce, you're up next. Bruce has worked out a quantitative flow test. In doing so, he found some things that don't work.

DR. DAVIS: So now you can find the flaws in my experiment. The reason I'm doing that is you're talking about tests that affect a few thousand maybe a Million people. This test is directed about 10 Million times a year so it's significant in terms of economic impact.

What I've been working on for many years and it's finally to the point of ready for FDA clinical trial testing is looking at CD64, the high affinity FC receptor on neutrophils and trying to make it a better assay for infection or sepsis detection. This is a shot from the coast of Maine in the early morning.

We published this data to support this idea in the mid 1990s. Since then many other groups have found supportive evidence of the fact that this is a better mousetrap for detecting

infection. I won't try and convince you other than this list that it's worth doing.

When we started we used Abe's beads and I was all enthused about MESF units being the be all and end all to quantitating what we're measuring. This is basically a unimolar shift in the level of CD64 on neutrophils. Monocytes constituently expressed it. Lymphocytes are negative. We basically used a system where we expressed it in MESF units feeling that with this kind of unimolar population looking at percent positive was not the way to go with this measurement.

I don't know, Abe, if you remember this conversation but we were doing fine through multiple lots of beads and then we got a new lot of beads. They didn't correlate numerically at all. So I called up Abe and said what's going on. His response was what number do you want. I can change it. I realized from a batch a lot to lot point of view this is going to be problematic. Remember it turned out that you changed a PMT in your FACScan and that was the difference between the two. At any rate, it pointed out to me at that point in time that there may be some problems or issues with

that.

This is just some data to support our belief that it's a better way of looking at infection detection. These are all culture positive infected patients and showing how CD64 is elevated. Whereas one of the standard tests, neutrophil count, is normal in many of these patients.

Very quickly the other thing that validated our belief in this test was an assessment of patients coming to the emergency room that were blindly scored as to the degree or certainty that they had infection or sepsis. Then looking at these four groups from the perspective of what you might consider tests for inflammation or infection. The neutrophil count really didn't find much difference. Said rate not much better. This is band percentage of left shift immaturity index or regarding what you want to call it was slightly better but still not perfect. This is CRP. So you can see the imperfect separation with the current test. This is our CD64 using MESF units. At least to my eye, it's much better separation of the four clinical groups.

That convinced us it was time to set

sail to put a kit together and quit my job as a pathologist and see if I could make this go. In putting together an assay obviously you are going to have to look at precision and want any clinical test to have a precision level of C/V less than 10 percent.

In this game obviously we wanted to get similar normal ranges and be able to get the same answer off of any instrument from any manufacturer. We had to look at how issues of stability of the sample as well as pre and post staining with the antibodies. My experience now with clinical laboratories is if it isn't simple it's not going to work. More and more so that is true.

We needed to look at gating or cell identification issues. We were going to quantitate CD64 granulocytes but we also want to look and find monocytes and lymphocytes because these actually turn out to be our internal controls. If the monocytes aren't positive, somebody didn't add the reagent. If the lymphocytes aren't negative, there is another problem because they should not express CD64.

Then there were issues of how to really quantitate or express the values. As I said

earlier this is a unimolar population that all the neutrophils shift as a group that is in a subpopulation effect. Looking at percent positive is worthless in this case. We use as a first start the median channel fluorescence of this population.

I went through the problems we had at least early on with MESF beads. Another problem could be cost that it would contribute to the kit. The next thought we evolved to is using internal reference in other words lymphocytes to derive a quantitation. It turned out as we looked at this more and more that too had problems. What I'm going to describe to you is our hoping to be our ideal solution namely a single calibration bead for everything. I described how we use lymphocytes and monocytes for control.

DR. BRAYLAN: What was the problem -

DR. DAVIS: No, there is no problem with that. For the control it works beautifully. What I had looked at several years ago was the concept of rather than MESF unit quantitation was to actually ratio the median channel fluorescence and the neutrophils to that of the lymphocytes. It's a truly negative population albeit a biologic population. That actually turned out to correlate

very nicely with MESF units. These are just two different sets of data points. You can see there's a very high degree of correlation. Obviously the numbers are different but the correlation is quite good.

The more we looked at samples, the more it became apparent that in the real world of clinical samples there are problematic issues. Even though there's a correlation, there are samples that fell out. This is just one set. I only have frequency of about one to three percent of samples at least in the samples we were looking at. The problem is these are samples that did not lyse very well. So instead of identifying lymphocytes, we were identifying lymphocytes and red cells. Obviously the autofluorescence of red cells is less.

DR. BRAYLAN: Did you use 45?

DR. DAVIS: It was just added expense. That was one of the things. There are samples that truly did not lyse well at all. It presented a problem. What we wanted to look at then is can we use a single bead that can be identified easily by other properties than light scatter. What we ended up with after looking at blank beads and FITC

labeled beads and actually this is the first talk with no more QuantiBRITE. We used another company, Bangs.

This was on what I thought was an excellent suggestion of Abe's to use a bead and use red fluorescence to identify the bead and have it labeled with FITC which is the same fluorescent tag as on the anti-CD64. It additionally has the advantage that when this comes out with a bead it will be a NIST traceable quantitation. That's what we use.

As you can see here other than the outliers it correlates very well with the lymphocyte index. We now have it so that it's a no wash technique. This is another set comparing washed to no-washed showing it gets similar results.

Now we looked at the issue of inter-instrument variably. I don't have the answers. I only have the observations. Within our own lab, we had one FACScan in my lab, one in Ken Alt's lab and also a caliber. So we had three instruments to my mind of somewhat similar instruction or technology made by a single manufacturer. I thought great. We should be able to get the same results in that

hand. You can see with these set of samples that there is a clear bias when you compare the three instruments. So that didn't make me real happy.

Then we looked more rigidly at how we ran the assay and what we were doing. We then came up with the idea that we probably should turn off compensation. Probably compensation is one source of this error. The other is what the gain and high voltage setting were. As I look back at this dataset, the negatives were further down the fluorescence intensity scale on one instrument relative to the other two.

The other thing was so we're going to turn off compensation and we should probably develop a rule to how to set up the instrument. It turned out that this four plus micron bead that we use that has fluorescence both FITC and the spitfire red signal can be used to tell people how to set their instrument up of both light scatter as well as fluorescence in all three colors that we collect in this assay.

So we developed a rule that specifics aren't important of how to set up the gains and high voltage and light scatter settings. When we did that using the same samples we ran them on the

same three instruments. Bingo, the curves pretty much overlie each other with a very good correlation.

We've obviously extended that to look at more instruments. These are different clinical samples with the index as we now run it. You can see that they all on these three instruments overlie very nicely not only the three instruments but we also looked at what happens when you hold the samples an additional 21 hours and rerun them. Do we get the answer? They again overlie very nicely.

As time went on, new instruments came out. Beckman Coulter now has their FC 500 so we've now looked at most of the clinical instruments and get curves like this. It's not perfect. I wish I could say that the curve, you get exactly the same number independent of the instrument. But we are now at a level that the variation is somewhere around five percent between instruments with the same sample using this methodology.

Just to give you an idea of how we look at the data and potentially another source of variability which leads to the part where I want to bring the message that it's not just the

instruments, reagents and the beads. We have to pull in software. I want to show you how we do the data analysis first in what I call a manual approach using anybody's software.

We use WindList. We take the list mode data first, clean it up to get rid of what un-lysed red cell or what is used. The assay actually contains not only two monoclonal to CD64. The reason for two is that it increases the signal, the noise, a little bit better than a single monoclonal because the two clones react to different epitopes. For reasons primarily related to developing software that I'll show you shortly, we've pulled in a second antibody that's a monocyte marker, CD163, so that we can then use that as a gating parameter to clearly identify monocytes from neutrophils because in truly septic individuals this population will move up and merge with monocytes when looking at CD64 only.

We use this as a gating parameter for the cells. We have the red labeled beads here. From a manual perspective, we clean it up, gate on the cells, measure the CD64 as well as CD163 on the three populations, use the beads to get values for the green fluorescence and the quantitation is

simply a ratio of the 64 signal to the FITC labeled beads and get a ratio measurement.

Now obviously as you can see, there is some degree of subjectivity in this. To go into a naive clinical lab and say "have at it" is a formula for failure. So we then worked with the Vary (PH) boys and came up with an autogating software program which is a derivation of their QuantiCalc software where the software reads a list mode file and finds all the populations and spits out a number.

I'll just take you through how the gating works. It's an iterative cluster finding algorithm. First it finds the beads based on the red signal in a wide gate and identifying that. Then a cluster finding gate is set around that. Then it's quantitative. The software also allows the manufacturer, not the user, to set a value to these beads.

In this example here, we've arbitrarily assigned this bead a value of two. Remember that the cells are going to be quantitated and referenced to that number. So if we wanted to put in the MESF units, we could put in whatever it is, 55,000 and come up with any quantitation we want.

DR. SCHWARTZ: When you finish that, would that be a lot of beads?

DR. DAVIS: With every lot of beads. I'm going to show real data to convince you that it really works. We also could but haven't look at yet do a similar quantitation for CD163 on monocytes with the same ability to assign value based on the beads.

DR. MARTI: Bruce, for those of us who are nearly blind, I don't have an appreciation of the axis from this distance.

DR. DAVIS: First we start with the PE signal versus the red signal, FL3. That finds the beads separable from the cells. Then we look at a green versus orange or PE signal that then sets a tighter cluster around the beads. Essentially this is a wide gate and this step eliminates doublets. So we just look at the single bead.

DR. MARTI: So how would I sitting at 15 or 20 feet know in going from the first gate to the second gate that this is beads? What am I missing? The count density here must be what's changed.

DR. DAVIS: That's all beads from this point.

DR. MARTI: So here it's diluted down by cells.

DR. DAVIS: Yes, these are cells. These are beads.

DR. MARTI: So how many beads in 100 microliter of blood?

DR. DAVIS: The assay is 50 microliters of blood, 50 microliters of reagent. We add a lysis solution of one microliter and then five microliters of the beads are added before analysis.

DR. MARTI: And the beads are in what concentration?

DR. DAVIS: Roughly it's 5 times 10^6 per microliter. Part of the whole assay is set to make sure we look at a minimum of 5,000 beads for statistical purposes.

DR. ORFAO: Is the green fluorescence of the beads at FITC?

DR. DAVIS: Yes, it's FITC and its surface FITC. I hope it's similar in signal cells.

DR. MARTI: So at that point you have your reference standards?

DR. DAVIS: Right, this is the fluorescence reference standard. Note I also said it serves a role for instrument set-up as well.

Then we go through making sure the assay had everything thrown in that it should. We have again an iterative gating process by which the beads are excluded. We look at just cells.

Then based on the CD163 signal, side scatter 163, a wide gate identifies the monocyte cluster as distinct from neutrophils and lymphocytes. A tighter cluster then hones in on these monocytes and then it's quantitated in terms of CD64 as well as the 163 signal. The read out is again a ratio between this signal and the beads. In this case, the number is 41.96.

The software also has flagging capabilities such that the manufacturer can define what is an acceptable level of CD64 intensity on monocytes. A flag would go off if it were below whatever index is defined as acceptable. In this case it's well above. If it weren't, a flag would pop up for the user as they analyze the list mode file. They need to look at the issue of stain.

The lymphocyte population is similarly identified. First beads are excluded. Just look at the cells. Again side scatter versus forward scatter. In this case final lymphocyte population, get a tighter gate around that and again

quantitates CD64 versus 163. In this case you expect a negative population. We see a ratio of 0.41. Again there's a flagging process to the software such that if it were above a certain level a flag would go off to alert.

DR. MARTI: Bruce, in this one what is this population here?

DR. DAVIS: In this case, this is neutrophils. This is just scatter.

DR. MARTI: This is debris that you are trying to get rid of and this is what you want. Is that true? These are the limits so they are really tightening in this particular presentation. They are really up tight against it.

DR. DAVIS: In that, yes.

DR. SCHWARTZ: Are you sure?

DR. DAVIS: No, I'm not actually. I just did this yesterday. But you are right. This would be the lymphocyte population but the same thing would hold that you could end up with a number here that could then be used as a negative control population.

DR. ORFAO: Are these gates done in two Millimeters or is that the cluster analysis?

DR. DAVIS: It's cluster in two

dimension. It's an iterative process at least to my knowledge. Bruce Dagwell (PH) wrote it, not me. Then finally the other thing is to measure what we're trying to measure and that's neutrophil CD64. A wide gate excluding the beads to catch the leukocytes. Side scatter versus 163 identifies wide gate around mostly the neutrophils and then a tighter gate around them. It's quantitative. How you derive the CD64 what we call the neutrophil CD64 index. In this case, it's 0.99.

Depending on the software, it also has the ability to likewise send up a signal when the neutrophil value is above a certain level and this can be turned on or off. In this case, we've defined on the normals that anything below two is normal. Above two is an indication of neutrophil activation.

DR. VOGT: And so, Bruce, you choose your MESF bead to be about the same as the staining which you get from normal CD64 neutrophils.

DR. DAVIS: Yes, it turns out to be that. That wasn't the logic but that was the coincidence that it turned out to be.

DR. VOGT: 99 is a nice coincidence.

DR. BRAYLAN: So what's the purpose of

measuring CD64?

DR. DAVIS: Just it's there as internal negative control to make sure that somebody didn't throw in the wrong reagent or whatever. So we have monocytes as a positive control, lymphocytes as a negative control. If those rules don't pass it tells the laboratory that they really need to repeat the assay because there's something wrong. As I said, I've learned more and more that we need to have as many safeguards in clinical assays as possible.

DR. SCHWARTZ: Are those indices independent of the kit? Does an indices mean something?

DR. DAVIS: No, it's like a chemist saying this is the international --

DR. SCHWARTZ: Is the indices going to change?

DR. DAVIS: No.

DR. SCHWARTZ: So it is independent of the instrument and your kits.

DR. DAVIS: Right.

DR. SCHWARTZ: If the next set of beads that were made come up twice as high, what happens then? How do you adjust that so that your final

indices comes up with the same number?

DR. DAVIS: I'll show you.

DR. MARTI: Sounds like a manufacturing control to me.

DR. DAVIS: To validate the software, we've compared it to manual analysis by "expert" and that would be either myself or Kathleen. This is the correlation on 564 different clinical samples. As you can see, the software working in an automatic fashion just reading list mode files and not asking the user to do anything gave this relationship.

DR. MARTI: Those 564 samples, weren't those all normal?

DR. DAVIS: No, this is just out of the clinical hematology lab that very normal in this subset is probably one and below.

DR. MARTI: I'm assuming your index was 0.99.

DR. DAVIS: We focused on somebody truly septic or in septic shock. They're going to be way up here. I choose these because they're low positives and normals to be the strongest test of the correlation. Obviously if you peg a point up here, you will end up with 0.99 no matter what.

Again wanting to see inter-observer correlation, myself and Kathleen analyzed the same files independently and correlated it with what the software got. Again they all correlated fairly well with one another. This was what you've been waiting for, Abe. I didn't want to have it not show.

Again the real issue that we started with trying to solve or address early on was to minimize lot to lot variability between the assay. We have two different clones of CD64. So every time you create a new lot of each clone, you have issue of different F/P ratios. You have different bead lots although these beads are stable for many years. At some point, you're going to have to add new beads to the system.

In order to test that what we've done here is five different combinations of different manufactured lots of antibodies in different combinations. That's what these numbers are. Three different beads made of three different lots. One of the beads is not twice as high but one bead is about 60 percent brighter than the other two.

Essentially in this case we're comparing five different lots of reagent. You can

see the correlation of one lot of antibody and beads to the other four here. They all have great correlations or regressions but obviously they give different values on these samples. We have a problem, right?

It turns out that this interrelationship gives us a definition of how one lot relates to another. When we do the math and give different beads values to the software program, these are the same points with value assignments appropriately given to the beads. All five lots agree with each other.

DR. BRAYLAN: Why do you have such a big gap?

DR. DAVIS: They are just different samples. Here I wanted a 0.99.

DR. BRAYLAN: You have such a separation or correlation.

DR. DAVIS: What do you mean?

DR. BRAYLAN: You have a bunch of datapoints right here and then one or two over there. You are always going to have some variation.

DR. DAVIS: I always wanted variable results in order to draw a line. This is just 10

randomly pulled bloods and these two happen to be from somebody infected. But even down here, these fall in a line. It may not be 0.99 but I never look to see what it is. You do have a valid point but in doing this value assignment, you're going to want a range of values in here so that you can plot a line.

DR. VOGT: Bruce, what was the computational approach you used to assign your normalizing values?

DR. DAVIS: To me the problem with this whole approach is that every single future lot is going to be related to the first lot. So I started with giving the arbitrary value in this case of the beads having a value of two for this lot and then use this math here to calculate what to convert the other beads to. I don't know if Bob would answer but I would bet you're doing the same thing with the QuantiBRITE beads now.

DR. VOGT: Not necessarily.

DR. DAVIS: But in practice.

DR. SCHWARTZ: You're more on absolute numbers.

DR. DAVIS: But in terms of - you don't get lot to lot that good. That is a weakness in

this. The one problem I see with this assay is when I make a lot five years down the line, I have no proof that the values on that lot are the exact ones as where I started because one term drift can occur.

DR. SCHWARTZ: To that point, let's get a clarification on your first statement about how you can take any number you want. You clarify it but I'll give my shot here. I don't want people walking away and thinking I just made these numbers up. What happened there is like I said what you want meaning what did the last lot give you so that we can at least get consistent results. Then we went back and found out why we didn't get consistent results. Not that I made up these numbers to make it happen. He stopped buying from me too.

DR. DAVIS: No, you sold it to Bangs. I still buy.

DR. VOGT: In fact I want to congratulate you, Bruce, first on the wonderful effort overall but also on having the groundwork right there plugged into the operationalizing of the technology that has not quite caught up to the application here. But in about a year from now or

less than a year, you'll be able to convert these things to NIST traceable MESF units. From here on, that traceability should remain. Is that the case, Dolph, that there will be some mechanism for traceability if not in perpetuity at least?

DR. GAIGOLOS: I'll give it a shot.

DR. VOGT: So in that sense this is a wonderful example of doing the best you can with the tools that are available and leaving the door open to incorporate the more refined tools. Did you ever consider using the capture-type bead that would then normalize your consistency which might occur in the conjugates that were variant in F/P ratio or might be variant in refrigerators that went down for a weekend before anybody knew?

DR. SCHWARTZ: That was the first thing that didn't work.

DR. VOGT: So that's an important thing to know. I didn't think it would work that's why I asked.

DR. DAVIS: But using those beads can be an additional check on the value assignments whether they would be MESF units or whatever. That perhaps is actually a way to check for long term drifts in the calculations that may occur. It's

pretty well there.

DR. ORFAO: You didn't make any comments about the impact of sample preparation like washing or non washing, fixing, non fixing.

DR. DAVIS: I can. It has a huge impact. That's why you get different values if you wash or you don't wash. That's why our current assay is a total just add the stuff and no wash. We've looked at the impact of what kind of water is added. We're going to distribute this as 10X lysis buffer. We were concerned about if there was a pH change because obviously we're looking at fluorescein and we want to make sure the pH is where we want at 7.4 or thereabouts. So your point is well taken. That's why I evolve to making it so the user can't do anything wrong. We're not even going to ask him to make PTS right.

DR. ORFAO: -- would it still be easy to identify properly the lymphocytes?

DR. DAVIS: Other than when I make the slide, yes. There's enough autofluorescence or whatever you want to call it that they generally will be recognizable just by a side scatter versus fluorescent signal as well as light scatter.

DR. MARTI: Bruce, does the lysine

result in fixation or is it just an ammonium chloride?

DR. DAVIS: It's a proprietary mix with ammonium chloride.

DR. MARTI: The reason I wanted to ask was that when you compare the lysin not washed versus the lysin washed what happens to the index? I'm going to hazard a guess that washing causes the index to get smaller. The amount of CD64 that binds to the neutrophil.

DR. DAVIS: It does go down. You're right.

DR. MARTI: I wonder if fixation would stabilize that.

DR. DAVIS: It does.

DR. MARTI: Whenever you don't have fixation, you have all this on and off equilibrium stuff that's going on. Lysin not washed is one matrix and lysin washed, you're changing the matrix each time you wash if that antibody isn't fixed and it has some finite time to start coming off-

DR. DAVIS: That's one of the reasons why I think it probably is fairly robust because it's an unwashed situation. I showed before the fact that we got the same results 21 hours after

sitting around. Again we were concerned about if you ask people to wash, what are they going to wash with? What's the fixative going to be? I'm amazed at the number of labs that you call up and say what's the pH of your fixative. They look at me like I'm crazy. They just say you buy it, you mix it and you use it. For a fluorescein conjugate, it's something that we had to be concerned about.

DR. MARTI: Another question on the variation of your calibration curves. Do you call it calibration curves?

DR. DAVIS: Sure, that's good.

DR. MARTI: Since I'm assuming this is linear linear, we can now talk about Y equals MX plus B. One of the things that seems to be majorly changing there is the slope. What's contributing to the slope change?

DR. DAVIS: Differences in F/P ratio and also difference in the B value or the fluorescence of the beads.

DR. VOGT: Gerry, while these are interpretable as calibration curves, they are actually in a sense calibrators. I can't read those things. Or that B is a single point calibrator.

DR. BRAYLAN: So, Bruce, I'm still not convinced that by using a bead you get results that are better. You are seeing two or three antibodies in the same tube but to identify the lymphocytes and monocytes and create the index for the granulocytes. What are the disadvantages of studying that approach?

DR. DAVIS: I'll forget the beads.

DR. BRAYLAN: Yes, because using an extra antibody to identify the lymphocytes that would exclude the red cells and -. You just have CD14 for your monocytes, a CD3 -- CD 64. There is just five minutes between those three formulations you should be able to get the same results.

DR. DAVIS: The only problem is how am I going to make lot to lot adjustments. The same way and just tell people to use this fudge factor. You still have F/P differences with every new lot of antibodies.

DR. BRAYLAN: But you can compare the lot with the next one.

DR. DAVIS: But how do I correct that? I'm concerned about going to the FDA and say I'm going to let community general do the math on this and derive a value that they are going to report.

DR. VOGT: Well the biological standard theory groups - those are correct because if you are taking ratio of intensities between lymphocytes and neutrophils stained with the same conjugate, then whatever the F/P ratio any change in the F/P ratio would be --

DR. DAVIS: In theory but in practice I do see lot to lot differences.

DR. VOGT: The answer to the Raul's question then basically since it's a essentially a negative control, it's the same thing as a bead with no life setting up on a negative population. There is just too much variance. If you had a CD4 like positive calibrator in there.

DR. SHAPIRO: The lymphocytes are negative so this is like dividing by zero. Presumably if you are using any of the population as an internal control, monocytes would be a better population because under normal circumstances your CD64 on the neutrophils is less than it is on the monocytes.

DR. DAVIS: The problem is that monocytes also get regulated. Monocytes also have a fairly diverse expression in the normal population. That to me is - one of the beauties of

this assay is measuring something on neutrophils that normally isn't there to a significant level on the healthies. The healthies stayed and then goes up. Whereas monocytes really can be all over the place. I've looked at the correlations and that kind of ratio just doesn't work.

The other thing, Raul, to address your question as I've forgotten an earlier slide is that the instrument to instrument variability when you start referencing a dim or negative population creates a lot more bias than I can figure out how to deal with. Remember I showed that correlation between a caliber and a FACScan that wasn't the same. That was primarily about where you put the negatives and how those two instruments deal with it.

DR. ORFAO: Do you mean that you should place the lymphocytes on the post stain region?

DR. DAVIS: Yes, even doing this assay, I always did try and set it up so that it was well into the first decade and/or in the second decade. But even there it just doesn't work as well. Particularly in certain instruments it doesn't work well at all. I won't name those.

To finish up this where we are. I

didn't show a lot of this data. But the precision of the assay is below five percent and it's safely below 10 percent. Between instruments and our hands the stability of sample before the sample itself as well as post stain is less than 36 hours. Probably it's 24 is what we'll claim. It's now a lyse no wash, pH controlled assay.

We have a software approach that removes virtually any decision making out of the laboratory which is always good. At least our decided upon approach is a customized bead that we can trace back to the promised NIST standard that should make the Europeans happy. Right, traceable stuff? We elect to use the internal controls although someday we may elect to sell a control material, another product, where you can just make stabilized blood samples positive at various levels.

So hopefully you like our thinking. None of this could have happened without people to tell us what's wrong with the assay. Paul Guyre who developed the CD64 clones has been very useful. Other people have given me various degrees of encouragement and advice over the years, the Vary (PH) boys for doing a good job with the software

and some good beads from Bangs. This is my partner that makes the antibodies for us. Thanks.

DR. STETLER-STEVENS: At this point, we have coffee outside. Off the record.

(Whereupon, the foregoing matter went off the record at 3:14 p.m. and went back on the record at 3:46 p.m.)

DR. STETLER-STEVENS: On the record. Our next topic will be sample preparation.

DR. SHAPIRO: Bruce gave a talk about CD64 in a meeting in Sweden in 2000. While he was talking, I was writing. When you finished, I wanted to comment. The lyrics have been changed for scientific accuracy. (Beatles Song "When You're 64.") When you are healthy, wealthy and --, don't need to express, sites that tightly, bound to FC gamma so, you won't see them when you do flow. With inflammation neutrophils show what they lacked before. As things get darker what's the best marker CD64. Kits, kinetics can let you know when drugs take effect whether you scan or flow, this you can detect. When tissue's hot, infected or not, you will see the change. Antigen, inoculate it on Foyer's stick, hope that's not what's making you sick, more diagnosis, follow-up too, who could

ask for more. It's so specific, it's just terrific, CD64.

(Applause.)

DR. MARTI: If you get the words exactly right.

DR. SHAPIRO: They're in the book.

DR. VOGT: May I ask a housekeeping thing?

DR. STETLER-STEVENS: Sure.

DR. VOGT: I'll prepare a compilation of this stuff including those PDF files from the NIST papers so that people can download them. Any of the PowerPoint presentations that I've assembled on here, are there any of those that I should not include on that? Are there any proprietary or private?

DR. HULTIN: Mine is a whole slew of slides. This is just to lead up to after the conclusion slide because I was just finishing up last night.

DR. VOGT: Everyone will be here tomorrow morning so give me tonight to work on that so we can get a somewhat called master set and then tomorrow I'll have all of these sent on it and you can just pick them up and copy them from this

laptop. Anyone who doesn't have their laptops, we'll get a CD to you within a week or two.

DR. PURVIS: I need to change some of my slides.

DR. VOGT: We'll work with that.

DR. STETLER-STEVENS: The next section we need to start asking questions. We've been discussing problems and what doesn't work, what has worked and what we need to be aware of. Now we need to talk about what do we need to do. What can we design as studies? What do we know so far that works in various areas? What do we need to figure out and get down some lines of actions? Sample preparation and staining is the first step. We have to think about sample handling, panels, techniques in staining protocols. That's the agenda.

Bruce, you used a no-wash method. Larry, you mentioned a short time in which you could look at markers. But these probably depend on individual markers. Lance, you looked at things 24 hours later. So that's going to be a variable that depends on the specific biology of the cells and how they react within blood. But what are some things that we can conclude from what we heard

about sample handling and things like that?

DR. WOOD: What's an acceptable window for sample age in coagulants that are hand diluted?

DR. SHAPIRO: There's a literature on this. There's some literature on this. The literature addresses several issues. One is it addresses the effects of lysis and fixation and washing on fluorescence levels generally not done at the MESF level but certainly done in relative fluorescence intensities. There's an issue about the persistence of certain antigens with the storage time.

There are a variety of agents which purport to stabilize preparations for various periods of time. So there is literature that probably needs to be reviewed and summarized on this. There is also if you will, a gold standard. Since there are said to be some differential effects of lysis and other preferable steps on fraction of cells and there's differential cell loss. Soon to be that's another story of -- where you get differential cell loss of some lymphocyte subsets.

It is claimed that you may actually get some differential CD4 loss with lysis procedures.

The way to verify that is to set up where you use a cocktail of antibodies and you don't lyse and you don't wash and you gate on fluorescence. That would tell you the composition of the cell sample that is not subjective to any of these influences. Again that really has not been done in any systematic way but it could be.

DR. SCHWARTZ: Isn't this in the NCCLS document? That's what it's supposed to address, isn't it?

DR. VOGT: Basically the document is a compendium of these same questions, Abe, because as Howard said all of these things have been looked but probably none of them have been looked at systematically. We did as I mentioned earlier the fluorescent gaining experiment that Howard just described with CD4 and found it to be indistinguishable from the immunolyse preparation that we did for regular work. We were happy as clams and didn't want to look any further. All we've done in the NCCLS document with most of these preparative issues to say this is an issue and it has not been looked at systematically.

DR. LENKEI: I should like to comment because I was thinking in the last time that two

factors are very important for diagnosis and quantitation. One is class studying of cells. Alberto talked a little bit about this. Then the problem is if you wash in your procedure, how is this affecting class studying of cells. Because if the picture is not clean, then you get a lot of cells spread around. We know class study of cells is very important.

The other thing is working with multi-plex analysis. The last time I realized again the effect which -- was talking very much about, the importance of the affinity of the antibody because we only think that lysing and washing is bad. We derive our conclusions using a lot of antibodies. If we should select the antibodies that have good affinity then the problem of washing at least once, it can be the last. So affinity of antibody would be very good and how our procedures is affecting class study.

Then you arrive to the time of aged blood because it's affecting class study. So the problems of washing, lysing and so on is a basic procedure.

DR. STETLER-STEVENS: If you don't lyse and if you don't specifically wash, you run into

some artifacts due to individuals. The one that Gerry and Jan Nicholson and I were involved in where if you take some blood from some individuals and there's a definite population and you don't wash and you stain the cells with CD4 and CD8 they look like the co-express the two but it's just binding to another antibody that is not specific for the protein. It's the protein in blood.

DR. MARTI: Probably an antibody fractioning blood that you can adoptively transfer from certain individuals to people who don't have it.

DR. STETLER-STEVENS: And you can take the serum from these individuals and incubate other cells with them and make them do the same. So this is an artifact that you run into. I see it in HIV patients who say they're CD4, CD8 positive. This must be tumor and it's just we washed the cells and we run into a lot of problems.

DR. LENKEI: But you are speaking about washing or settling in the serum because they are stained. These are very important. There were some papers even from Janet Nicholson speaking about this problem. There are patients -- infectious -- and it's acting like a bad

compensation but it's not that. So we separate always the serum.

DR. STETLER-STEVENS: We pre-wash it. You do a stain and no wash.

DR. LENKEI: I wash in my laboratory.

DR. ORFAO: I think we are entering a subject that didn't come up clearly although there were some people mentioning it -- which is the biology, the regulation of the expression of the protein you are quantifying. It is sometimes I would say even more important than the technological issues.

So most proteins that are in the surface can be there, can be internalized, can be scattered. It's just allowing cells to function. Or sometimes by stimulating the function of the cell with a specific antibody, you are adding a monoclone. CD15 is a clear example of an antibody that makes cells change expression of other things in a very short period of time.

Many of these proteins which are in the surface it can leak, it can be shattered and they are in the serum. If they have another protein in another cell that they bind to, you can see unexpected stainings.

DR. FISCHER: That's a major problem in looking at certain markers because their ligand becomes effective. In fact sometimes both sides can actually be solubilized at least in the plasma. You just mentioned, yes that's a problem with autoimmune patients and that's even more of a problem in autoimmune patients because we certainly it in systems we're working with.

You really have to take care to know the biology of the system before you can work with anything. For us it just makes sense that we always have to prep the cells. You can't just drop the antibodies in and lyse them and expect to get reasonable results. It just doesn't work for the systems we work with.

DR. LENKEI: I was thinking about this effect. We are staining samples on the table. Then they put the blood and they add the monoclonal antibodies for 15 minutes. Then it's now sodium azide in the tube to prevent physiological processes. In my laboratory we separate the serum. We stain as soon as possible but always in our procedure we set at the bottom of the tube 50 microliters of the staining buffer which is part serum and the sodium azide. So in the stain at

least first --

With this procedure we are on the safe side. I want you to know from the specialists is that small amount of sodium azide which is added to the monoclonal antibodies soon after to block the physiological process. This approach which we have at my laboratory we get always, even in bone marrow samples from transplanted patients, a very nice plastering and no problem with high background or other things. That is costly because time is costly and then you stain for 30 minutes and we lyse and we wash once and we fix.

This theoretically is a good procedure because if you stain on the table without any sodium azide the risk is that the physiological processes are going on and you are very much depended if the lab technician has exactly 15 minutes on the table or 20 or 30 minutes your picture will be totally different.

DR. MARTI: Alberto is shaking his head no about the sodium azides.

DR. ORFAO: Given the values in the sample some of these processes are still able to go on especially if the protein is expressed already or the enzyme that cuts it is there.

DR. BRAYLAN: Do I have five minutes to make a long comment?

DR. MARTI: Jean-Luc and then we're out of it.

DR. BRAYLAN: What we heard today, we heard many presentations from individuals who somehow have measured it, the expression of certain antigens and correlated those findings with either clinical, biological phenomenon in a very elegant and very informational way. The techniques those individuals used to come up with those data we would probably find the same. I don't think there's any problem in whether they are trying to do the right thing or the wrong thing. But they showed using that technique, that it works we should follow that regardless of whether we are measuring exactly what we think is the measurement.

Measuring the true antigenic density on the other hand or expression on the individual cells on the other hand would be something that now we are requested to do by clinicians who feel that they need to know exactly how many antibodies -- site per cell. There are in certain tumors that they can apply certain antibodies for treatment which is a different story. I've been trying to

point that they know something in a very accurate manner. I want to emphasize that. We don't know how to do that. I investigated the literature. I wish somebody could tell me exactly how to do that.

I can just give some data that I have when I began working with CD20. Because of cohesions that we all know, they thought it would be very interesting to look at CD20 computation.

Before you can get into the points, I took the CD 20 unimolar one to one PE and I looked at the instructions. The instructions said that you need to use a certain method and exposure to the antibody for about 45 minutes at room temperature.

Because I wanted to have that as a routine test with all the other incubations that we do with the other antibodies listing the same times and same temperatures, my first question was would there be any difference in the binding of these CD20 PE antibodies. When I used the directions that the manufacturer was telling me to use versus my approach which is 15 minutes on ice. There was close to double the amount of sites when you do it at room temperature 45 minutes than when you do it

on ice 15 minutes.

We went on to then study other parameters. I can tell you in a nutshell that temperature makes a huge difference. Of course times make a difference. Concentrations of antibodies make differences. As I mentioned before we never reach it like that, with CD20 at least.

My question is we can do all kinds of preparations and we can study parameter of binding cells but is it really legitimate that you think that what we do in our testing in our laboratory under the conditions that we use. We extrapolate those findings to actually what happens which is the question that the oncologists have. Am I going with this CD20 reagent that they are using which is different from the CD20 reagent that we use to the patient something that we can predict in the laboratory situation? I don't think we can do that.

If we are going to measure true antigen expression on the cells, I think we're going to have to define first what we mean by that in what conditions. What is the actual absolute number of receptors of determinants on the cell surface? We have to define the conditions because there is

tremendous variation depending on things that are simple as the temperature and the time. I did not look at the lysing situation with all the other issues that are discussed in here.

DR. MARTI: Jean-Luc, did you want to make your comment and then we'll come back to that?

DR. D'HAUTCOURT: Yes, I wanted to make a comment a little bit earlier regarding the quantitation and also the relation with wash and no-wash. I disagree with that if you don't wash the cell you lose cell resolution especially with dim cells. But of course there is a lot of explanation but one explanation may be the reabsorption of the essence by the other. When we perform the computation of FITC in presence of two or three other markers to make a subsetting for quantitation especially if we use PE and B55 and B57 (phonetic). We have a lot of PE molecules in the suspension and the reabsorption of the FITC is contained too. So it's an important point to decide if you wash or not or if we use FITC in presence of other molecules to make computation.

DR. STETLER-STEVENS: I think that multi-colored flow is going to be a problem in quantitation but if you're going to use this

clinically you can't use single colored flow. You just can't do a clinical specimen.

DR. D'HAUTCOURT: Because if you use single fluorochrome for the quantitation you can not equalize the setting. If you don't equalize the setting, quantitation is set.

DR. STETLER-STEVENS: So multi-color analysis is going to be a difficulty but we have to do it.

DR. BRAYLAN: The other thing you're not mentioning is that you really need to combine that with a different antibody. Once you start doing this --

DR. MARTI: Let's not kid ourselves that the quantitation was hardly out of the bay. Why did two and three go the way it did? What is B-cell gaining? What is T-cell gaining? What is CD45 gaining? That cat's already out of the bag. The horse is out of the barn on that one. The same thing is happening with at least at the level of four and five color. It's not going to stop. So we have to figure out how to do quantitation in the setting of multiple fluorochromes. I'm sorry.

DR. SHAPIRO: We do and again everything we're discussing if we're going to get

anywhere with this meeting and with anything in flow design there is a great variation in biology for sure. We are trying to get a general framework to approach a large number of biological problems. The best place to start from is to just consider the factors that may be involved in the approach to anyone of these problems. Because in any individual case some of them are going to be important and some of them are not. A physicist or the people who do the physics problems you get in college physics text books, you are good at those because you eliminate all but the two or three factors that are important when you solve the physics problem.

In biology we never have that luxury. We have to deal with however many problems may bother us. So here what we should be doing is making a list of the problems because then when it comes down to, okay, we're treating somebody with a particular antibody and we want to know whether the density of the corresponding antigen on the tumor cells affect slightly the response to the antibody. Then we go to what are these cells? What do we know about them? There is a checklist because at some point we're going to have to look into all of

those factors before we get an assay which we can be sure is going to give us relevant answers. Basically we don't want to get bitten in ass by some factor.

DR. VOGT: If I may suggest that we make that a matrix and have you all tell us on the Y axis we would have a list of factors. Along the top column headers would be the list of markers. What are the most important markers now that are considered to be important? CD20 sounds like a good candidate to start with.

DR. MUIRHEAD: CD52.

DR. LENKEI: Then put it in an inventory of problems what can be eliminated.

DR. SHAPIRO: CDs are no. You are going to have 250.

DR. LENKEI: Not the CD. I should say that we will have a basic understanding. All of us. What are the problems and what can be eliminated exactly as how I said?

DR. SHAPIRO: Before it's eliminated, is it a severe problem or medium problem or something we should check on?

DR. LENKEI: I should analyze the situation now perhaps also with some grading of the

gravity of the interest of this factor on the standardization of measurements. If it's quantitation or it's not quantitation. Anyhow we have to have it in it and we have to be able to compare our results in multi-site status.

DR. FISCHER: I think along the checklist ideas is you have to look at it and say is this going to be a case by case problem versus something that can be addressed as something overall and not just if it's a medium problem or it's a hard problem.

DR. LENKEI: Because this is the benefit. When we have the overall then I can go to specific things.

DR. STETLER-STEVENS: Some things that will end up being caveats, for example, antibody choice, I think we should probably use the same clone that you are treating with in antibody therapy. But maybe in some antibodies they're all equal and the therapy doesn't matter. Whereas in others it may be very important in which case there would be a caveat that one must validate that you can use a different antibody from therapy before you can proceed in using that for testing. What else do we need from the antibodies? What are the

types of things you have to think about?

DR. BRAYLAN: To minimize these factors I wonder whenever possible can one use an internal biological standard. For example we abandon the idea of QuaniBRITE because of the issues that I just mentioned. Just simply using our normal B-cells to establish a ratio of the CD formula expression of my normal CD20 cells over the tumor population. The conditions are the same. The technology is very similar. Other than the expression of density of the surface -- should be very small.

DR. VOGT: Did you do in a sample, Raul?

DR. BRAYLAN: The same sample if they are present. Even if you don't have it on the same sample you can take one sample. In other words minimize all these issues of azides and lysines and whatever because it would be a double standard assuming that the normal -- that would minimize a lot of factors.

DR. SHAPIRO: That's what we did with DNA. It's the way we had chosen to do DNA with the IN index. So you have a plant biologist who are doing taxonomy and then your DNA picograms.

DR. BRAYLAN: That's true. The problem with some of this is that not every system has internal knowledge.

DR. STETLER-STEVENS: But it's difficult with something like CLL with a white count of 180 to find --

DR. BRAYLAN: That's what I'm saying.

DR. STETLER-STEVENS: But the idea of having the normal control, do we need to run normal controls simultaneously?

DR. D'HAUTCOURT: We use quantitative predictors.

DR. LENKEI: Not for CDs.

DR. SCHWARTZ: If you wanted to go after A1.

DR. LENKEI: Not if they are in marker. I would go for general rules not for markers.

DR. MARTI: Do you just want to look at this?

DR. SCHWARTZ: Yes, they may be different for the specific tests. If they are, we've used a lopsided matrix, is it important to worry about how well we do it on a CD64 as opposed to a 69? How long is the cell stable for before you actually do the analysis? But generate a

matrix like this because in certain cases it won't matter if it's 15 minutes on ice or 45 minutes depending on the PD of the antibody.

DR. LENKEI: No, because I don't think anywhere you will be able to confuse the clinician with indications for each antigen.

DR. SCHWARTZ: This isn't for the technician. It's for the people developing the assay and then have the specific things for that assay which will work across laboratories and instruments.

DR. STETLER-STEVENS: That's going to be when you are developing the assay.

DR. SCHWARTZ: Yes because when this individual thinks you can at least -

DR. STETLER-STEVENS: But that's something that we can deal with.

DR. PURVIS: You have to evaluate this on every assay that you are setting up.

DR. MARTI: That's right.

DR. PURVIS: There's no way around it.

DR. SCHWARTZ: You have to do it and there really aren't that many things you need to quantitate yet. So it's doable at this point in time.

DR. VOGT: My argument for putting up this specific list of markers was that's easier, not that it's harder. Without a specific set of markers, I don't think you'll ever be able to come up with a time of incubation that is fully adequate without being overly long.

DR. STETLER-STEVENS: I don't think that we can come up with a time of incubation and say this is what we recommend because next year they are going to have a new antibody.

DR. VOGT: Right and it's going to have to be looked at again.

DR. STETLER-STEVENS: You are going to have to do each time. So what we can come up with is you need to look at the time of incubation and it has to be standard.

DR. SHAPIRO: What I had in mind was that we want a list of the factors. It would be helpful if we say these are the quantitative assays that we know most and we know best about. We can say that we have something for our CD38. We have CD64 and we know that there's extensive experience with things that worked. We can say to the technicians that this is how they are done if you are trying to do something new.

The idea is for as Mary Alice has said we're talking about a treatment protocol, then it would be best if we use that antibody. Thus far the manufacturers may have been reluctant to release the antibody. When you set up the protocol you say we need to do this study, let's have the antibody so we can use it.

DR. SCHWARTZ: The other value of this matrix is once we throw up the things we know looking at it we may be able to generalize it in an intelligent way.

DR. STETLER-STEVENS: We can have an historical perspective of what's been done and what works and what were all the factors with a full understanding that whatever you're studying none of those may be appropriate.

DR. SHAPIRO: Correct.

DR. STETLER-STEVENS: By showing that there are differences or even by an internal study where we would demonstrate analysis, there are big differences. It's based on time of incubation, antibody clone but maybe washing and no washing or whatever. If we show that there are differences, the thing is there can be a list of things of what's been done and what you have to do before you

can have a standardized procedure.

You have to be aware of using the same time period for incubation, same antibody function. That is the recommendation we come up with by having the search perspective showing differences. We can say these all have different conditions for staining and preparing the cells therefore you have to determine each one of these conditions for your new assay. If you are trying to reproduce what others have done, you have to use exactly the same X, Y and Z. You can't take this and use it as map of how to do some CD52 CamPath. It's not going to work. But if you take this as map to figure out what I need to do look at for CamPath.

DR. MUIRHEAD: How did you decide the average flows were just minimum? That was the road. - could be an extremely useful one.

DR. VOGT: Some of it can be teased out in literature. You can review this paper, I forget, there are a number of co-authors there. There are obviously some markers that were cloned and conjugate sensitive and others that were rather robust. That, of course, is another line on there, is the reagents themselves. One approach to this is to go back and look at what's in the literature

but I don't think we'll have these preparative methods so well.

DR. SCHWARTZ: But we can contact the people who did them and get them from the authors.

DR. VOGT: Well that might be good. In some cases they might have looked at those and come with something without reporting that they noticed that it didn't matter how long they stained for or they may not have looked.

DR. FISCHER: Unfortunately this kind of thing may not get into the papers because what they are more interested is the science rather than in the technique because that's for another journal and of course they never write and it stays in their lab all the time. We have several in our lab technical things that I don't think have ever made it out.

DR. BRAYLAN: Things that we use for the standard of incubation period.

DR. VOGT: Right, most of it isn't.

DR. BRAYLAN: As far as I know and I looked into this when I saw my results to see if anybody has controls to be looked at, different incubation periods, temperature, there isn't single paper that addresses that.

DR. MARTI: You might do that.

DR. SCHWARTZ: Because it's going to be different for every clone.

DR. BRAYLAN: Right.

DR. STETLER-STEVENS: For any antigen that hasn't been done.

DR. BRAYLAN: Define all the conditions.

DR. SCHWARTZ: But if we had these detailed matrix then we could start bringing out generalities based on some facts.

DR. MARTI: You can already go to the literature like Bob started out this morning. No offense intended, Bob, but there are at least ten more papers that show CD4 values in the literature, all independent care relieved articles. I'll bet if you go back and look at the methods where it's available, you'll start to get a feel for the breadth. The same thing is true for CD20. You have some values, the guy in New Jersey has some values, I have some values. There are several papers right there. I bet not one of them is identical.

DR. LENKEI: I think we have enough results now published to come to some conclusion.

DR. STETLER-STEVENS: You think that there are results published where someone has looked at time and temperatures. CD20 is probably not the right one to do it with since we know that there is this plateau. There may be other antigens that we may want to study.

DR. LAMB: CD22.

DR. MARTI: I think CD4 is the positive control for these studies.

PARTICIPANT: But there is no use for them.

DR. MARTI: Then choose one that everyone would agree with that they didn't get 48,000 or 50,000 and you couldn't immediately say what did they do wrong.

DR. DAVIS: My point is not to be so much facetious about that but to some degree we ought to be thinking about the hardest cell to do like the monocyte.

DR. MARTI: Or dendrite cells. Anybody want to tell me immunophenotype of dendrite cells.

DR. VOGT: One of the points here that -- emphasizes and I'm just reemphasizing it. I'm reading a paper by George Yannasee and others where they had this nice compilation. But I don't think

in that paper that they ever said that they looked at titres to determine if they were at saturable staining let along looking at these other things. One of the first questions I would have on any system is can you demonstrate saturable staining. If you can't then maybe you should scratch that off the list and say maybe we can't quantify it. Of course if it's very important then maybe you should work harder to see why you can't get saturable staining.

DR. FISCHER: Do you mean to go to a FAB instead of -- molecule? Maybe you need to switch clones?

DR. MARTI: You can put saturation on your list but I'm standing here thinking which is more important saturation or specificity. The reason I think about specificity is because of non-specificity. The idea of using cold antibody with blocks.

DR. VOGT: That's another thing too. Maybe the right way to do this is not -- staining and look at an endpoint but rather to do a quantitative titration. It might be the right answer does not lie in the endtube. But the answer to this lies in several tubes.

DR. BRAYLAN: That's fine but we can standardize it.

DR. VOGT: Then we can standardize that. That's the old dose response which generally gets you more information than a single point anywhere.

DR. BRAYLAN: There is another issue with saturation and perhaps somebody can correct me if I'm thinking wrong but remember in a form of leukemia it is very common that cells with the antigen that you are looking at are three times or ten times the size of the normal lymphocytes upon which the agent was tested and titrated and declared to be saturation of that.

So anyway the population that could vary from one percent to 90 percent are huge elements. The control to saturate that with the titres that you use for normal lymphocytes you never reach the saturation. That's another issue that we have in cancer that has to be dealt with. Perhaps blood is as good as anything else.

DR. STETLER-STEVENS: If we are going to do some studies there should be, from a practical point of view, a B-cell antigen because CLL samples can provide enough cells for multiple

laboratories to study. It's easy to do. Whereas you can have somebody with a very high white count and you can easily get a sample that you can distribute.

DR. SCHWARTZ: If we do these studies, what do we expect to get out of that? What's the goal?

DR. STETLER-STEVENS: We can answer Raul's question about what happens with different times and by demonstrating that if you for example use different antibody clones, different times of incubation, different lysis, you are variable all over the place. People will stop and say wait a minute. Do you mean I have to look at all this stuff? They stop and do it the same way. I can stand up and say I know it's going to be different but does that matter to anybody out there in the community. Well prove it.

DR. SCHWARTZ: So are you going to have 15 people doing it 15 different ways and get different answers and say that proves it? It doesn't.

DR. STETLER-STEVENS: No.

DR. SCHWARTZ: And is one person going to do it 15 different ways?

DR. STETLER-STEVENS: More than one person is going to do it 15 different ways.

DR. SCHWARTZ: They all get the same answer the specific way.

DR. VOGT: That has to be an iterate process because it becomes geometric and it is geometric and becomes intractable real quick. One thing is we've tried to do this on two model systems, one that is relatively straight forward and has been well worked out. I'm wondering if a hairy cell leukemia might be an easy target.

DR. STETLER-STEVENS: No, because they have cytopenias. You don't want to bleed them too much. You just can't get a sample.

DR. VOGT: Okay bad idea.

DR. STETLER-STEVENS: They are hard to get the cells. I can evaluate hairy cell leukemia as a single site because we get many each week. We get maybe four hairy cell leukemia specimens a week.

DR. VOGT: How many markers on the hairy cell would be amenable and useful for quantification?

DR. STETLER-STEVENS: CD22 would be very useful since we treat them with anti-CD22.

DR. BRAYLAN: For quantitation or --

DR. STETLER-STEVENS: Quantitation.

DR. BRAYLAN: But there's a protocol.

DR. VOGT: A therapy.

DR. STETLER-STEVENS: We are looking at CD22 anyway. So I can do hairy cell leukemia. They come in. They're fresh.

DR. MARTI: What are the antibodies that you're being asked to quantitate?

DR. BRAYLAN: 20, 22, 19, 33.

DR. STETLER-STEVENS: Syntax CD6.

DR. BRAYLAN: 52, 25.

DR. MARTI: I heard rumors about 23.

DR. LENKEI: But not 36 or 33.

(Inaudible.)

DR. VOGT: Now the other thing about 38 is that we have good reference values on 38. If we were going to try to do 38 in a way that did not require the CD34 biologic calibrator that's probably a factor of the problem and we would know if we were getting the right answer because there's a history out there. So 38 strikes me would be a good model system. It's seems to be useful to you all.

DR. MARTI: I assume that 33 is setting

of ANL.

DR. LENKEI: Yes, but then it's not to be quantitated on BCLA. Then you have these two for differential difference.

DR. MARTI: I'm just trying to get a census of the group of which ones are being asked to quantitate, 22, 20, 19, 33, 52.

DR. BRAYLAN: Many of them have already monoclonals in the --

DR. LENKEI: Then you have to take CD103 -- because it's the best one. It's the new BCLL7.

DR. MARTI: Is it being injected and fused?

DR. LENKEI: No.

DR. MARTI: It was my understanding that these are ones -- Is 19 still in use? It was conjugated to Ricin. It's pretty toxic to the liver. 22 has replaced 19. I know there's one other one missing which is Hu1D10. That's the class two. That's the study of the two sites. One is at the NCI and one at University of Iowa.

DR. FISCHER: We're being asked to use 19 in some of CD23 trials because of the fact that it seems like the CD20 is not available for us so

we're being asked to quantitate the 19 for B-cells.

DR. TAMUL: Also CD117 seek it.

DR. VOGT: And Gerry, I think therapeutic targets are primary. In those cases where there is considerable prognostic value, 38 is not a therapeutic target, I guess, but it's very useful.

DR. MARTI: No, I left it in because of that.

DR. STETLER-STEVENS: Why can they do it in psoriasis?

DR. MARTI: Psoriasis is whatever the receptor is for the tumor necrosis factor alpha. That's where with the monochrome antibody. I'm sorry I've forgotten its name.

DR. STETLER-STEVENS: They've done some other too.

DR. MARTI: There are four or five that have been infused but I've forgotten their names.

DR. SHAPIRO: Do we know within the next leukocyte differentiation workshop which is here in Adele and is being headed by Heddy Zola who is fairly old hand at quantitative immunofluorescence.

Does anybody know off hand what's

planned for that in terms of antibody quantification? Steve Shore probably knows. That's something that we should look into in the sense that if we find out about it now we might be able to get a lot more information by the time the workshop actually rolls around than we would have otherwise.

DR. MARTI: It's interesting that you should mention that because I'm thinking of one particular workshop where it was involved. It was one of the first ones he was involved with. That's when MESFs were used to form the antigrams. But even prior to that workshop at the previous workshop, raw fluorescence intensity numbers on there were challenged when that number was used.

DR. SHAPIRO: I remember that at the Boston workshop that was in 1995.

DR. MARTI: That was probably MESFs. The one before that was -- That's a good thing to think about. The next leukocyte differentiation.

DR. LENKEI: I don't think it was general because being bright and medium. It was two blue site classes.

DR. MARTI: There was actually MESF numbers.

DR. LENKEI: Yes, MESF. But before MESF

DR. MARTI: So this is the list of antibodies that have clinical targets. It must be INBs (phonetic) and must not be approved yet.

DR. PURVIS: No, they're still going.

DR. MARTI: They are on-going trials. Actually I had a table made just before I left and I couldn't bring it with me but there are 15 or 16 monoclonal antibodies that have been approved for in vivo use in humans. I'll see if I can sneak back in there.

DR. VOGT: Is that a public list?

DR. MARTI: It's not proprietary.

DR. SHAPIRO: Did you know MOBT stands for Mother of Biotherapeutic.

DR. MARTI: I don't think we're going to shed any more light on the kind of conditions around everything under the name of sample preparation staining. One of the things on the list was panels. This really isn't a panel. What were you thinking under the terms that we're thinking of phenotyping panel just in the setting of four reagents or more?

DR. STETLER-STEVENS: Yes, multiple

reagents and how are you going to do it? What have we decided that we need to do? What I've heard is we decided that we need to review the literature, come up with a listing of what's been done and how the conditions vary. Is that correct?

Secondly we need to actually sit and several people need to study time of incubation, temperature. All of these things determine saturability, all of these factors with a specific antigen and report it because that's not in the literature.

DR. SHAPIRO: I would imagine that the antibody manufacturers, especially the ones who are making kits, would have some of the information that speaks to this.

DR. STETLER-STEVENS: I would imagine so.

DR. McCOY: As I listen to this whole approach, that we really need to tear it apart a little bit better. We've been looking at a lot of trees. How I see the forest is, there are three areas. One is the medical necessity/economics of the clinical environment. What do you really need to do? What's the easiest way to do? How are you going to get reimbursed for it? All that. That's

one area.

The second area is the biology of the cells that you need to look at. What's going on with the cells, with the markers on the cells and so on? Then the third area is the technical aspect. What do you need to do the quantitative flow to quantitate marks from just a fairly technical standpoint? Now all these areas interrelate. They interrelate probably in very disease-specific, marker-specific way. If we are going to start tearing this apart, we have to look at all three of these areas and have them interrelate.

DR. LAMB: Can I add one thing to that? It is developmental therapeutic. Not necessarily clinically utility but also used in developmental therapeutics when you're designing antibody-based therapy, quantitative flow could be very useful. Even if it doesn't ever reach the practicality of a clinical laboratory, it's highly useful in that setting.

DR. STETLER-STEVENS: There's going to be indications of reimburseability. There's what we can get reimbursed for now. I expect flow to be around in ten years and what people got paid for 10 years ago compared to now. If we demonstrate that

something is useful, it will be ordered and will be needed. If we never demonstrate to do that, if we demonstrate that it's not useful, it will not be ordered and will not needed.

DR. BRAYLAN: I'd like to second that. I believe the medical necessity and the economics will be clear if we have good models and good technical aspects that show some biological symmetry on --

But unless we have that, we should show clinical guidance that we can do something for them. So it's going to very difficult to try to justify the medical necessity. We don't have a list. If we had a list, at least we could see if it has any medical implication.

DR. ORFAO: I think that that's true, but not always. If you look at how treatment develops, you will see that even molecularly target treatments. When you go to the patients, the response is heterogenous and you have to look for a explanation. Clearly when you use monoclonal antibodies for treatment, the first explanation you would expect is that it depends on the amount of protein that your cells express. So that's why you are required after the treatment is there. In this

case, necessity is not coming because you proved -- it's coming because there is a therapy that people is using that is targeting a molecule that they think is development.

DR. BRAYLAN: Right, at this present time, we are requested to provide some sort of indication of how much that protein is expressed. We do it now with pluses and minuses or with intense medium -- What we are trying to do here is try to make that expression of the cells a little bit more elegant, not necessarily more scientific but more elegant and comparable among laboratories.

Once that is in place, then the commissions can say, okay, let's take all of these patients that had so much of this according to this that you can see they responded differently than those other patients that had a different number. But at the moment, they cannot do that.

DR. ORFAO: But if you look at the history especially of breast cancer and monoclonal antibodies against -- they have stopped the research in the sense that you only get treated patients who express a certain amount of protein. So you are already bias -- so you cannot analyze the clinical value afterwards. What I fear is that

the same will be done quickly for the lymphomas.

DR. BRAYLAN: That's true. That's true, but if you have data, you can go back and determine if the treatment is pretty much the same across the board. So you could potentially go back and do a study.

DR. ORFAO: But if you look at CD20 therapy, you got a paper saying that it was useful in multiple myeloma. And you want people looking for the explanation.

DR. BRAYLAN: I understand. But, Alberto, remember what he said initially when he started to talk -- that what one laboratory says is positive, another laboratory says negative. At least some sort of standardization would allow us to prepare cells in the laboratories, and then for clinicians to say does this mean anything.

DR. ORFAO: I think it's very, very important to have that problem researched.

DR. MARTI: We really haven't been discussing this topic of quantitative flow in terms of standardization.

DR. LENKEI: Standardization is the most important.

DR. MARTI: Many of us feel that the

principles of quantitative flow that we lust or seek after, that -- that the primary by-product of that would be standardization.

DR. LENKEI: Yes, and then coming to the studies, the first thing is to first think about the protocols so that we standardize as much as possible, because otherwise they would be without any meaning. So I think the most important is to use experience, our experience, to design protocols which take into consideration what we know now about the standardization in this topic.

DR. MARTI: In order to become and remain an active participant in the AIDS cohort studies, what is the plus or minus value between CD4 and two test tubes in order to stay in that program and be designated an NIAID approved lab -- plus or minus what?

DR. HULTIN: Five.

DR. MARTI: I thought it was two.

DR. HULTIN: That's for reproducibility. It's four. But across labs, it's plus or minus five.

DR. MARTI: So plus or minus two in the lab, across labs five. I stand corrected. Thanks. What should be the similar goal here, with

quantitative flow? It should be some type of -- I'm going to say this and you can shoot it down, but it should be a calibration curve that's plus or minus. If it's beads it should be plus or minus two percent or less. If it's antibody binding beads, plus or minus five percent. I'm just saying that for the sake of discussion.

DR. SHAPIRO: That's two percent of what though? What are you basing this on -- the calibration curve plus or minus?

DR. MARTI: I'm sorry. The fit of the beads to the regression curve.

DR. HULTIN: Did you decide that --

DR. MARTI: No, we haven't, but I just used that. If we are going to say that in your laboratory, plus or minus two percent of the same antibody in different tubes -- if that represents a level of reproducibility at the percent determination, what would you accept?

DR. HULTIN: I've taken a sample and run it ten times. If it's a tough marker, I've seen it vary by 10 percent, just the same two,just collecting it again.

DR. MARTI: I guess I'm not making myself clear.

DR. SHAPIRO: No, you are making yourself clear. It's when you asked the question. But the way he started asking the question was, what is ACTG as for the way of reproducibility. And the thing is that ACTG -- there are statisticians who decided on what they should ask for in the way of reproducibility. And we don't have any of them here. Those are the people who should be defining what we need to do for this assay, because --

In fact there's a very nice study which --I think Molo Gorman is probably the first author on it -- looking at the biometric imaging device for CD4 counting versus flow cytometry? And that study is done differently from practically every other comparative study of medical methodology that has been published to date, in the sense that everybody else would take the two methods and then publish the correlation curve between the two methods.

And it was Becky Gelman who did the statistics for that points out, that's ridiculous, because if you've got two machines that are designed to measure the same thing, of course you're going to get a good correlation curve. And

what you need to determine the experimental biases -- there are always biases -- is to devise two kinds of tests. And she devised those. All her tests are devised, and that kind of thing serves as an example.

And if you're asking -- to answer the question that you've asked, about what do we need to shoot for in terms of agreement, once we get this from one lab to several labs, what kind of agreement do we need to make this useable, you really need the input of one of those statisticians.

DR. MARTI: Well, I think we can get that. To complete the thought, because of my simple-minded approach to this is, that I think the parallel to getting plus or minus two percent on CD4 positive cells between tubes is that, in order to do quantitative flow you need a calibration curve. And whether you display the calibration curve this way or this way, all I'm trying to ask is: How do we measure the acceptance --

DR. SCHWARTZ: You're doing -- what is the acceptable performance of an instrument is one question which we used to use to qualify a laboratory that even participates in a quantitative

survey, because if they didn't have an instrument that worked, we knocked them out. But the real question to be parallel to the ACTG is -- in terms of either intensity in MESF units or ABC, hopefully the antibody was all the same and then you could go to ABC by knowing the F/P ratio -- how do they compare across things. And I don't think you're going to get better than 10 percent.

DR. SHAPIRO: I would agree.

DR. SCHWARTZ: And people have bitched about 25 percent when we've done our things, and Rodica did one for a year for five countries or so, and she got around 25 or so percent. And that's -- you know, compare that to radiotracers and that's pretty damn good.

DR. MARTI: If you are a new lab just setting up in West Virginia, and you want to do quantitative flow, what would this group require?

DR. SCHWARTZ: Again, you should use Mandy's thing of -- that's a learning curve --

DR. MARTI: Okay.

DR. SCHWARTZ: -- and you expect that lab after the sixth or seventh time to come into wherever -- the rest of the mark. Now, we're looking for an absolute number, and from the

experience of the people who have run these kinds of things for quantitation, we've usually gotten -- at the absolute best was five percent when we controlled everything, with only five labs that we beat over the head to do it exactly the same way.

On the more open surveys, we've gotten -- on really good agreement -- 15 percent, and it's gone up to as much as 25 percent. There hasn't been the things that were run properly in 50 or 100 percent. And this is both in ABCs and MESFs.

DR. MARTI: So if I go into a CLIA certified lab and I'm asked to do an inspection to see that this laboratory can be certifiable for quantitative flow, what am I going to be looking for? When I open the book of SOPs, how will I know?

DR. SCHWARTZ: CLIA is -- Performance Agreement Across Labs is another. That's the CAP Labs. You are asking two different questions.

DR. MARTI: Again, to me, that comes back to a calibration curve and performance characteristics.

DR. LENKEI: Yes. So I would look at the quality control of things. To me that is one of the basic things. After that, I probably would

look at the reproducibility of result; after that, the coefficient variation. Because we are doing that. We validate our methods at our laboratory. So in my condition, I would look at the entire -- for good antigens to see how is the consistency of results.

DR. MARTI: So one of the guidelines from CAP is that I split a sample in my lab and take it to your lab and we run it blindly. Then we sit down and look at the data together and see if it matches.

DR. LENKEI: It's more than that, because you have to choose robust protocols in order to be able to do that. So instrument is one thing. If we want to run something, then we have to select the most robust protocols. What this needed to do is to improve the reputation of quantitation for clinical application. In that case it should be a good selection of protocol survey of agencies, and what do you know about standard of instruments, and then do it.

DR. ORFAO: But I think it's important to note that, in a certain way, people were using semi-quantitative approaches by flow in a day-by-day basis. And that's why you see the minus, plus,

plus-plus, and plus-plus-plus. And this means that sometimes it might be that really useful information is not a median value or a mean value. And it might be over or below a certain value.

So I think it's important also to have this information in mind that not in all situations -- let's say, if it's a very high value, it might be -- it's clear that that would be responding to treatment. If it's very low, it's clearly below the cut-off. So it's also important to notice that there is like a window, which is much more important than the --

DR. SCHWARTZ: What you have to understand is the window, and not do it subjectively.

DR. SHAPIRO: But years back, when the clinical interest in quantitative study first came up -- and I don't even remember how many years ago it was -- Alan Landay had me make the rounds of four or five labs that were doing the good stuff. And they all had -- they were all using the same antibodies; they were all using FACScan -- and so they were all given FITC MESF beads, your FITC MESF beads, European MESF beads to run.

And what it came down to is -- if you

picked out a cell type; I looked at them in different labs -- and they were making no attempt to define a window analysis or quantify anything. All they were doing was running the MESF beads, you know, daily or weekly, or whatever. And what I observed was -- if you looked at, say, where these CD4 cells came out, you know, if they came out between peaks two and three of the MESF beads in your lab, it came out between peaks two and three of the MESF beads in another lab, which basically backs up what I've heard.

It's saying that intuitively there is a semi-quantitative base of flow data that is really there. And so -- and in many respects, it almost gets harder when you try to make it more quantitative.

DR. SCHWARTZ: Except when you get to the next lot of beads, unless you make it quantitative, you're screwed.

DR. SHAPIRO: Right.

DR. ORFAO: I think the established approach is the comparison with the normal cells you have in the -- So both cells stain for the same marker. It would be what Bruce was showing for CD64. Instead of having lymphocytes which were

negative, you would have a positive controlling site of something from the same antigen.

DR. SCHWARTZ: That's a good -- to make the solutions but you can't.

DR. HOUTZ: It seems like, properly done, you might demonstrate validation. But if you were the inspector and if it was my laboratory, I would need to demonstrate that there were criteria being followed, that there was specific rationale for that criteria, that there are particular guidelines with respect to sample prep.

I don't think we can all agree, I mean with respect to sample prep on, you know, a particular lysing time or incubation time, and so on. But I think that, you know, there's a lot of this methodologies were developed here by manufacturers, or a lot of manufacturers using investigators' data. Investigators had their own assays. But I think, you know, we could establish specific guidelines in terms of fixation. You will see a loss in terms of ABC values. You know that you'll see changes with respect to temperature.

I think that if we essentially can agree on specific guidelines and basically make a specific recommendation on those guidelines, that a

number of these factors are going to affect your results. Then I think that we could be at a point where we could begin to establish a certain set of guidelines and perhaps sort of a method validation framework that customers -- not customers, investigators -- could use which would be useful for them in establishing their criteria for whatever quantitation assay they are going to perform.

DR. LENKEI: The problem is that fixation can increase the fluorescence of some antigens and can decrease the intensity of others. It's really a tremendous amount of factors which are interesting. So I think we have to have some basic approach, some basic tools, to go farther.

DR. FISCHER: Everybody's using the same protocol and the same antigen.

DR. LENKEI: Yes.

DR. FISCHER: Then it won't matter whether it increases or decreases the fluorescence in the long run because we're all using the same protocol.

DR. LENKEI: Yes. Exactly. Exactly. But then you have to have standardized protocols and to use the same antibodies, because probably

this is the confusion. You have to use exactly the same protocol, exactly the same antibody conjugates in doing the same protocol, to perform exactly, to try to adjust your instrument. And then you have the same results. If you think about multi-site status or comparison of results in the laboratories, then you have to find the conclusion at the end.

DR. TAMUL: I'd like to just comment on this and what she's saying. And I'm basing this on two factors of my experience. One is just as a salesperson going into quite a few different laboratories, both clinical and research, throughout my territory and, in some cases, other parts of the country. The second is evaluating submissions for the ASCP flow qualifications, which include asking for detailed information on how the instruments are set up, calibrated, what antibody QC they do, what specimen QC and process control they do.

I can almost guarantee you that if you took everybody's protocol in this room and put it up against one another for, whether it be for instrument set-up, whether it be for antibody QC, there are going to be differences. It's not

standardized.

DR. ORFAO: I think also if there is a paper saying that if you strong CD20 or you have over a certain number of molecules of CD20, that the patient will respond. People the next day will be trying to reproduce that.

DR. TAMUL: And I think we also have to recognize that perhaps not every flow cytometry lab that even does immunophenotyping is going to be able to successfully and accurately yield a quantitative result. It's something else to think about.

DR. ORFAO: Also I would like to say that -- my impression in the consensus meetings is that we have many opinions but few data.

DR. TAMUL: Yes.

DR. ORFAO: And that's typically a problem. I would favor selecting one of these areas and go for it. Certainly you will find and you will control for many variables that would be useful for other.

DR. LENKEI: Yes, because it was our project in the group -- Not so many consensus papers based on expression, but experiments. Because it's very easy to talk about things.

DR. ORFAO: Are you talking about consensus but, you would say, not of opinions --

DR> LENKEI: No. Consensus --

DR. ORFAO: -- for scientific data?

DR. LENKEI: Yes. For scientific data.

DR. STETLER-STEVENS: Can we say -- at this point, can we make a statement and be certain --put this in writing -- that you must, in order to have intralaboratory comparisons, you must use the same incubation time, incubation temperature, antibody clones, and have a list of things that you must do? Can we say that, and do we have the data?

DR. SCHWARTZ: Intra?

DR. STETLER-STEVENS: Interlaboratory.

DR. SCHWARTZ: You need to do it intra first.

DR. STETLER-STEVENS: Intra too. So, if we can make this statement and we have the data, then that is the statement to make. You have to use the exact same conditions for it, and list them. And we can come up -- If we can come up with a consensus as to that --

DR. BRAYLAN: We can't come up with a consensus of doing this to do the experiment.

DR. STETLER-STEVENS: Yes, you can.

DR. BRAYLAN: But we need to test it.

DR. STETLER-STEVENS: The consensus on what needs to be looked at.

DR. VOGT: One intermediate position on this is that we need to construct an editorial, for instance, or a meeting report or however you all want to approach that, and to point out that at this time it's hard to interpret the literature even though it's out there, because of these factors and that there is a group that is now going to systematically look at -- on a case-by-case and factor-by-factor basis -- as model systems. Not that we can look at every case and every factor.

But we can certainly look, just as Dolph has done with fluorescein. You know, it took some years to get that one thing down to where, I guess, it's close to rationing.

DR. STETLER-STEVENS: But then we need to look at again, if people already have looked at it.

DR. VOGT: Yes. There is not in the literature now enough information to compare across the values that have been reported for binding capacities. I think the only exception is the CD38 data.

DR. STETLER-STEVENS: However, if you have inter-laboratory -- if you have data, research data, that you have done, demonstrating that I can make different values based on different times of incubation -- or, if I do it on ice or at room temperature. And if we have that and I can show this, then I think we can state pretty much you have to have the same conditions in order to get the same answer. If we had data that's already been done. Do we need to go out and redo it if Rodica has done it, for example?

DR. MUIRHEAD: Well, you know, she's using a different clone, and you may --

DR. MARTI: The only thing that you don't have to do is if two labs are doing it identically right now, and I might even say -- I might even say it was peer reviewed and published. That might be one you don't have to do. Is anyone aware of such a situation?

DR. MUIRHEAD: I think you need to go back to what NCCLS states.

DR. STETLER-STEVENS: In CD20, you have to use X conditions. What I'm saying is you can say that you have to think about all of these conditions when you do quantitative flows. You

cannot assume that your temperature is okay. If we have data showing that it varies according to -- if you differ times, you differ this, you differ clones -- then we have data indicating that you can't use different clones carte blanche. You have to stop, study and validate -- for each antigen that you study -- the methodology. And you use the same methodology to get the same answer. That's what I'm talking about. I'm not saying --

DR. SHAPIRO: Assuming that in some respects, there's stuff in the literature that says incubation time, license conditions, temperature, the clones you use -- all of these things will affect the measurements you make. And so, since we know that that's true -- I mean, the question is, are you asking that somebody -- since we know that that's true, we assume that if we control for all of those variables in designing a protocol for use in multiple laboratories, that we will minimize the effects of all of those factors. And I think that that's not a bad assumption.

If you are asking, do we need to actually design the study that shows that, for this particular thing it makes a difference, I don't think we need to do that. I think that just from a

practical point of view we can say, let's just stipulate that this is how you design a protocol.

DR. BRAYLAN: I think we all agree there are different conditions that make for different results. We don't have to redo that.

DR. STETLER-STEVENS: Yes.

DR. BRAYLAN: The problem is to agree on what? How are we going to agree on temperature or something else?

DR. STETLER-STEVENS: That's going to have to depend on the antigen. You study it. You can't say that what you're going to use for one antigen is going to apply to another antigen.

DR. BRAYLAN: No, but what's the gold standard? I don't know that. The problems I have with CD20, I don't know which approach is better and works.

DR. MARTI: That's the problem. You don't have a gold standard. I still think the gold standard for positive control is CD4.

DR. VOGT: There is no question that's it's true and that we should use that.

DR. MARTI: If you can't do CD4, you got a problem.

DR. BRAYLAN: But you said CD4 is --

DR. STETLER-STEVENS: When you're looking at B-cells, you're going to have different problems. It's going to always change. I think it's going to -- we're not going to come up with -- If you made everything for this amount of time, at this temperature, with this clone doing this match --

But, what we can define is, how do you determine how to set up your quantitative flow.

DR. SCHWARTZ: It's all based on where you get a plateau, and you have to accept the criteria of saying you have to have a certain quality of that plateau to even to do quantitation. What it takes to get to that plateau is up to you. Some people could do it on ice for an hour, and other people can do it for ten minutes at room temperature, and you will get the same plateau.

DR. BRAYLAN: Are we going to accept the plateau as being the best reference?

DR. SCHWARTZ: If you don't, you can get any answer depending on any condition. So if you do not get a plateau, you better not do any quantitation with that answer.

DR. WOOD: What I was wondering is, we're tackling this from the top down. We're

looking at the thing, talking about the things that are difficult to do. What about starting from the bottom up and finding out where the gray zone starts, the basic things. Let's run beads. Can we agree on beads? Moving there to simple cell models, and moving on up the line. Where does it become gray? Where do we move from saying: yes we can do full interlab type comparisons, to: well, maybe we can almost do it here, to: this is the point where we stop. And we can't do it yet.

DR. FISCHER: That's true because, before you can start comparing any methods, you gotta first start with something Abe has advocated for years, and that's instrument setup. What are you doing to use to set your window of analysis?

DR. LENKEI: We have that also in the second project. And I hope we will publish it soon. I am guilty, as Bob says. I have all the results, one year's work with the same window for finalizing, the same one as I told you, and it showed that the variation can be very much effected.

But what I think is important if we select B-cells, we can do this again, based on all our knowledge, and selecting the basic protocols

according to our knowledge today.

DR. BRAYLAN: But then you have to go back to the instruments.

DR. LENKEI: Yes, but --

DR. MARTI: I want to go back to Jim's comment that starting with beads you might all think, oh yeah, we can do, looks pretty easy. Do you know when Myron Waxdol was doing proficiency testing and he sent out beads? As: Well, now, this is something that the lab won't have to stain. They can't possibly screw this up. All they gotta do is put it in a tube and run it. Well, if there were two peaks, you can count on somebody missing one. If there was three, you can count on somebody missing two.

DR. SCHWARTZ: Twice I did CAP studies provided for 700 laboratories and asked how many peaks were in this thing. Sixty percent got five. Twenty percent got four. And it was because they didn't set the window of analysis and their key was off-scale.

DR. LENKEI: In our study, three laboratories couldn't set the same window for finalizing during one year. It showed up that they --using the local window if they had good

experience with that, they could -- they had good results -- But, again, and bad -- with the -- presenting the final data for finalizing.

But, yes, it's important to have the best protocol to look for these. And they were analysing the variations -- but, no harm done. I used the beads 10 years now, and my conclusion is that, as we know, we have to use both beads and cells as calibrators, because it can be a problem with space and I have very limited space in my system. Because beads can lose their fluorochrome.

And I remember once with my quality control and with the best system with quantum MESF, that I obliged Becton Dickinson to change and to revise my instrument according to the publication from Canada, that when you use these functional parameters then you can detect instruments which have bad logarithms -- amplifiers. And I did it and they changed it and we never got good functional parameters. The technician from the system got so furious that he reversed the amplifiers, and still it was the same thing. They were the beads.

So I want to tell that. The concept is very good. But if you don't have problems for one

year, two years, but we have to be aware that we can get into problems with beads that we get. So, in my laboratory we have a quality control in which we have beads and we have cells vague. And the cells are CD3, stained with 4 especially for calibration because calibration has more problems even now. You have to detect the problems, especially the three of these, which are so important.

So we have the same window for finalizes on both sides -- and for calibration. We get the same when we do it after each instrument service. We look at the window for finalizes and we look at the reproducibility, which is quite complicated. But then we are on the safe side. So to set the window, we have both CaliBRITE and we have QC windows, because one of them can be wrong, and then you know that it's not this one. It's the not instrument. It's the beads.

And then for other things also we have -- we are on the safe side. So I want to say that beads are very good, but we have to be aware of this.

DR. MARTI: Kathy.

DR. MUIRHEAD: Someone has proposed an

averaging approach, and I understand why. Because another possible way to do it is that -- today there are a bunch of people in here that are interested in B-cells, for one reason or another. Okay? Now, I also happen to think that this is a group of people who are very expert and interested in quantitation. I'm sure that each of them in their own laboratories has worked out something that they have found to be reasonably reproducible, and they have their reasons for having chosen whatever it is that they have chosen.

If you could get four of those people to share protocols with the other labs and learn each other's protocols, and say, okay, how do we do and share samples, and what -- and say, okay, you are each going to have some kind of variations. And that is, I think, the fastest way to find out which variables seem to influence the results most, and which ones give you the most robust, if you like, agreement -- even within that small group -- and help focus some of the -- okay, what are the key variables for the system?

Now, I agree with you that you can put together a list of variables that are likely to influence the outcome. But I think a faster way

that -- I'm in great sympathy with Alberto's -- that we need data. Start with -- pick a system. It won't tell you what to do for every other system but would it establish a precedent that there was a way of getting to a consensus by exchanging protocols and data.

DR. SCHWARTZ: I mean, you can do that by making a matrix, a checklist against the labs, find out where the differences are and then have the people run an exchange and things like that. But we have the roadmap -- as NIH likes to call things now, roadmaps -- on what to do. Because that's the data of how each of these laboratories do things.

DR. STETLER-STEVENS: We've already determined that. Lance, you've talked about what you've done, have validated in your system. And we have systems where he's able to get good quantitation in more than one laboratory. That's done with a marker and antigens. Period.

DR. MUIRHEAD: That's using a single protocol, okay, and that's what we've found out day-to-day would be reliable.

DR. TAMUL: Actually it's surprising -- and you and I are thinking very much alike.

Because when I wanted to say originally when Mary Alice brought out that we don't have published data, part of the reason is that we are totally ignoring a huge amount of data out there, that's not published because it's not coming from academic laboratories who have the time or the motivation or the push to publish.

There are huge sets of data out there, some of which could come from the large reference labs. Norman and I have talked about this before at some other meetings. There are a number of large reference labs out there that have developed some very standardized protocols. If there was a way for them to share that without infringing on confidentiality and competitive issues, there's a huge amount of data there that could used.

DR. PURVIS: That's what's difficult, because there are experiences that people have that are major factors. We have already raised these here several times. Each system you are going to end up looking at all of these variables to make sense of what you can do. There are some systems that you may not be able to completely saturate, but if you choose the concentration and an antibody to use it at in a standard protocol, you can

reproducibly quantitate a value. And that's, I think -- I think that's what we have to do.

I don't think we can get to a point where we're always going to be, you know, this is at saturation. But if I can get consistent reproducible results -- and clinical trials are really what I think is going to introduce this. These are on-going now, and we're really seeing the utility. Yes, there's proprietary information that's involved in that. And I would love to share all of the study data that I have, but I can't do that. I can show you some of the stuff that we've developed that we've talked about in general senses in the past. But I don't think we can go, you know -- I'm going to give you my protocol and here's -- you go off and run it.

DR. BRAYLAN: And it's to go back, because most of us -- we have hunks of data too, with 20 years in a very standardized controlled manner. We can go back and --

DR. TAMUL: But that's what I'm saying is, that many of the larger reference laboratories have more data on more patient specimens in a shorter period of time where the methodology was likely to be more standardized.

DR. PURVIS: Across laboratories with multiple sites and multiple controls.

DR. TAMUL: Exactly. If there was a way that data could all be somehow put together without identifying the source of the information, that could just say, okay, this laboratory did it this way and found that these variables work. Laboratory B did it this way and found that these were important. And put that together in some sort of meta-analysis, it could then be loaded on top of each other and eventually the things that are important would come out.

DR. MARTI: Jean-Luc, comment.

DR. D'HAUTCOURT: Because we don't have enough data. Maybe first basic experiment is, and because some of use believe that we can receive an order of a clinician to make a computation on some antigen, maybe someone of us only believe in the old one. Let's suppose that in a few weeks we receive all the same request of the clinician and make a quantitation of the CD20 in these patients. If we perform this result in dispute and we don't have an consensus --

DR. MARTI: Let's say if we had a sample on a normal person and we could share that

with several labs for CD20 determination or would you want the same sample on one patient?

DR. D'HAUTCOURT: We can perform a mean like this before. Let's take ten different number of patients and we get normally after 10 or 20 we must get the same mean.

DR. LENKEI: CD20 has a much greater variability than CD4 anyhow, even in normal patients. Not to talk about variability of CD20 nature in patients which have been demonstrated, we can talk about that. One thing would be for the future when we do these experiments to work with stabilized blood for this type of experiment as a common substrate. We can talk about things because even if we have many results normal MV and ME and others if they are not published, it will take five years to publish that. We can use our experience to design probably something that would be useful for the future.

DR. STETLER-STEVENS: Norman, you have work-up conditions for several antibodies. Are they the same for each antibody? So there is not going to be any standard protocol, period. So bury that idea.

DR. PURVIS: But we started a table up

there and taking the matter of fluorochrome and right now these are factors that must be evaluated each time you go through developing an assay and let's not worry about writing down this CD and this CD. Then as we develop assays, the question is going to be whether my assay and your assay we can look at and come up with the same conclusions. It may depend on the antibody clone that I choose versus your choice. If we're going to look at this, we're going to have to be able to share our data in some way and come up.

DR. FISCHER: Gerry, between you and Bob to FDA and CDC you ought to have a way to put this maybe even on a secure database so that maybe somebody's data that they don't want necessarily out among the general public, if we are all bound by the constraints of having been involved in this not to release the data to the general public, but share the information on a secure network where you can actually go in and fill in the table under Norman's name and he does an experiment. Then he fills in all these criteria that he's figured out is best for this particular antigen. Maybe I've done the same experiment, I fill my results in there and they are a little different. Then the

two of us can say let's figure out where the differences are.

DR. PURVIS: And this is where Kathy was going here to find out whose methodology is most reproducible within multiple labs. If we have four methodologies, I've got my methodology, you've got your methodology, she's got her methodology, and I run yours as well as mine on the same samples and then we compare.

DR. SCHWARTZ: With this availability you will be able to use it without having to do a whole bunch of mess of quality, etc. It's also like a blind study because he's not holding your hand doing it.

DR. D'HAUTCOURT: Let me make a comment. If we expect a very large variation in individuals, is it logical to follow the goals of every standardization for quantitation?

DR. LENKEI: Yes, because when you detect the variation --

DR. D'HAUTCOURT: Yes, if the individual variation is so big.

DR. HULTIN: It's much bigger.

DR. LENKEI: Yes.

DR. HULTIN: It's okay. CD38 has a

huge variation. You can go from 500 to 1,000. But if you have 2,000 that's a lot different.

DR. LENKEI: It's not standardized. You don't have a statistical significance.

DR. STETLER-STEVENS: The two action items out there, one is to list from experience what you have to do when you start out. As consensus guidelines you have to look at this. You have to have the same this and the same that. Because, that shows people what -- A second one is to take protocols that people have and to test for big use, what we see as an important use in the future and to test them at multiple sites and to have those of us who are willing to follow somebody else's protocol.

DR. SCHWARTZ: The third step down, these lists are now what people's protocols are on some chemical website where that would the data of how things are done and then you design experiments to validate what looks like the best one from those lists.

DR. STETLER-STEVENS: How do we know what the best one is?

DR. FISCHER: That's why I said if you list them up there the ones that you are doing and

the way you are doing it and we put them out there then we can all look at this.

DR. LENKEI: If you would ask people on the same blood sample to produce their individual probables, some standardized cytograms. For example we will select criteria which are important for diagnosis. Then you distribute to the stabilized center and then you ask them to send to you the cytograms and the results. Then you don't need to test again all the protocols in the laboratories. You can compare the results. And it's enough. Because I know when I work on my paper with experience, I look at the figures, and then I can tell. I look, I read, I rely upon this paper or not because some papers are based on bad premises from the beginning so I should select. We can get a very good consensus.

DR. STETLER-STEVENS: You can't tell by reading the paper that methods is going to be reproducible.

DR. PURVIS: There's too much data that is missing a lot of the literature so that you can't reproduce what they have.

DR. SHAPIRO: And it's somewhat intentional too by many researchers. They put in

just enough to satisfy the reviewers but not so much that they give away their intellectual proffers.

DR. STETLER-STEVENS: That's true too. So it would be good to test two or three protocols identified in several laboratories and you work within your laboratories. If I use one protocol, I get the same answer when I do it in duplicate, triplicate, etc. Then I use another protocol and I know there are problems in reproducibility within that protocol to start with. Between laboratories, this is something that we could do, not for every protocol in the world but for a few that could work. Not everybody in the world is quantitating antigen expression.

DR. PURVIS: A lot of systems that probably will not make the clinical mark. They have intuitive pharmaceutical companies that they study the EK, BD, all of this information. We have to be smart and choose uniformly to one goal.

DR. MARTI: Bob has a comment.

DR. VOGT: Of the things that you can control there's a lot but there's good control over the instruments. Now the instruments are pretty good in the way you can set them up and use them is

relevant and consistent. Currently you can control a calibrator. You can send everyone the same calibrators. The third thing you can control is the reagent used for staining. In other words, what I'm saying is the prep method seems like the biggest source of variability between labs in a practical sense. Whether it's a source of variability in the final answer is we can get that. You can get an answer to that question. That's not a terribly hard question to answer.

If for instance you send the same normal blood sample somebody with enough T-cells to count to every laboratory that wanted to participate with the same calibrators, the same reagent and maybe more than one reagent and said run your prep method and report to us your CD20 and not by the way just a single mean answer but send us your list of vials so we can look at your percentiles and see the spectrum of distribution standards, then you would know how much the prep method introduces to the variability and the measurement of CD20 in normal whole blood. Given that people like Norm and Raul have looked very carefully at this particular system, we would tend to think that if everybody got answers that were

close to theirs then that's probably good news. If there was a spectrum of answers, you would try to figure out who was doing the thing that was most carefully worked out. If you try to get everybody's method in doing everything, who is going to keep that? Who is going to organize it? Who is going to keep it up? I think some good laboratory data that limits the number of variables as much as possible and I think prep method is probably going to be the variable that's going to be the hardest to get people to change. People are used to doing their particular method.

DR. BRAYLAN: Unless you show a better way.

DR. VOGT: Unless you show that it's important. The only way that you show it's important is by locking down all the other sources of variability and showing that the prep methods have to be standardized. That could be done in six months.

DR. STETLER-STEVENS: I have a suggestion. Gerry and I were just whispering up here. I'll say it openly and people can refuse to join in, but Norm and Rodica, you both have strong opinions in this area and have data in this area.

Would you be in charge of a subgroup to determine protocols to be studied for an antigen. I'm willing to work on it. People who are willing to volunteer and to do what needs to be done to be laboratories tested can identify themselves to you.

DR. LENKEI: I should like to take Alberto on that because he was running the experiments in Europe and has such a systemic eye. So I think the three of us.

DR. ORFAO: But only for some time because I'm very busy now.

DR. BRAYLAN: May I ask a question? How are we going to support this?

DR. STETLER-STEVENS: We have to get some funding. We do have offers for supplies such as beads and things like that. Yes, we have to work on that. We also have to have people who decide what are we going to study and what protocols. We can't try 14 protocols. We can't try everybody's protocol. I'm happy to let these two people decide.

DR. LENKEI: We have all the same approach.

DR. STETLER-STEVENS: I'm willing to do what they tell me to do. If we can get other

people who are willing to participate and do what they come up with, somebody else will have to work on the support and getting the reagents together. If you can come up with suggestions for protocols.

DR. PURVIS: My experience is that the vendors are very willing to provide antibodies. Every system that we have gone into clinical trials with, I call them up and say I'm studying this. I want to be considered. You send me your antibody. In almost every case I have had every vendor's antibody sent out to me and I can sit down and I can do my own.

DR. ORFAO: Just from pharmaceutical companies who are treating antibodies?

DR. PURVIS: Some of the time what we do is they will provide us if it's a clinical trial and they're asking us to develop their assay for them, then yes, they have a monoclonal that they are using that's one of the first things that they will do is provide that to us for the clinical trial.

DR. STETLER-STEVENS: You are going to come up with a recommendation for how to look at -- quantitation. Does anybody want to be cut out of that? Does any manufacturer not want to be

considered in coming up with a protocol?

DR. MARTI: I can't understand why IDAC would even, given the variation on the determination of CD20s, I don't know why IDAC would want any flow lab in the country to make a determination on CD20 if it depended on whether they were going to use their therapeutic monoclonal antibody.

DR. BRAYLAN: Do you think we could get their antibody for testing?

DR. MARTI: That part I don't know. I heard rumors that they don't share it very well.

DR. SCHWARTZ: If we do this approach, you are going to get three opinions which is a very limited number of opinions of how to do it. I would like to be able to have a committee be established with maybe these same people just list the checklist, all the things that should be done. That should be the first thing to find out.

DR. STETLER-STEVENS: That's was the first action item.

DR. SCHWARTZ: They can that today.

DR. STETLER-STEVENS: But the next thing is what are we going to do after that?

DR. MARTI: That can be done tonight.

I like that.

DR. SCHWARTZ: It will be done tonight and bring it in the morning. Whoever wants to help can help them. The second thing is I'd like to get for all those checklists if you pick a particular marker that you want, get everybody you know to be able to fill in this checklist.

DR. STETLER-STEVENS: It's not going to work like that. It's going to be different from each antigen. And that I don't think we can do.

DR. SCHWARTZ: But we need to find out what people are doing now, what antigen they are using, what times, what temperatures.

DR. STETLER-STEVENS: What the different numbers in it wouldn't help.

DR. SCHWARTZ: But if you see a pattern, how many people are doing it for three hours? Nobody. But if there's a range than these three people with experience can say what is the consensus of all the methods that have been tried.

DR. BRAYLAN: But there's no consensus because we haven't tried it.

DR. SCHWARTZ: That's what I say.

DR. STETLER-STEVENS: That's the data we don't have. What we do know is the things that

are important. We don't have it.

DR. BRAYLAN: No, it's the data. I don't have any choice.

DR. SCHWARTZ: Nothing is correct in that. If you have all the protocols of people who have actually done the testing, then you can make an intelligent guess.

DR. STETLER-STEVENS: I don't think you can make an intelligent guess. You have to test it.

DR. SCHWARTZ: This is the problem. You are establishing your hypothesis to test.

DR. STETLER-STEVENS: I'd rather establish the hypothesis what to test based on their knowledge and their sense of experience.

DR. SCHWARTZ: -- experience in what is the hypothesis.

DR. STETLER-STEVENS: But we're going to focus on one antigen.

DR. SCHWARTZ: On one antigen.

DR. STETLER-STEVENS: Right. But the list that we can make is important. Temperature is important. You can't just do it. These are the things that we can all get consensus on. That's what we can come to consensus on tonight. There

are just certain things that are important.

DR. SCHWARTZ: And even things that aren't you should be able to list.

DR. STETLER-STEVENS: But I bet we'll fight over exactly which one is right. If you don't say it's important but mine's right not yours.

DR. BRAYLAN: There's no gold standard so it will be very difficult to determine what's right and what's wrong.

DR. SCHWARTZ: It's not about what is right and what is wrong. It is what is reproducible.

DR. BRAYLAN: What works.

DR. STETLER-STEVENS: We don't know that.

DR. HOUTZ: The fact that it's important is your input. That's your guidelines.

DR. STETLER-STEVENS: And what we can just say is that we know from experience is that the time that you incubate is important and it changes your result.

DR. SCHWARTZ: Right now, let's ask these three people how long you incubate a CD38 and at what temperature, just those two factors.

DR. MUIRHEAD: It depends on what antibody you are using.

DR. MARTI: 30 minutes action at room temperature, CD38.

DR. PURVIS: We use 20 minutes.

DR. MARTI: 20 minutes at room temperature.

DR. LENKEI: I was saying if it's protocol, I saying at forty degrees to be on the safe side.

DR. MARTI: How long?

DR. LENKEI: One hour.

DR. MARTI: So 30 minutes room temperature, 20 temperature room temperature.

(Inaudible)

DR. STETLER-STEVENS: I think we would agree that there are factors that if you vary them it's going to vary your result. We can't agree on what's the right way to do certain things. What's reproducible? We can study what's reproducible. We all have experience that if you change certain things you get a different result. That we can say is a caveat.

When you are setting up to do quantitative flow you can't just choose a

temperature for incubation out of the air. You have to determine the optimal conditions for a list of things.

DR. SCHWARTZ: But these three people who did it on their own all got three different answers.

DR. LENKEI: If we could choose three protocols.

DR. PURVIS: We don't want to quantitate CD38.

DR. SCHWARTZ: All three labs do.

DR. PURVIS: I haven't gone through the extensive study.

DR. LENKEI: No harm done. We have a lot of experience in this field. Dozens of patients.

DR. FISCHER: Answer your question. Whose clone do you use? Whose clone do you use?

DR. LENKEI: You use the same clone.

DR. FISCHER: This is what I'm saying. He asked what temperatures you used and maybe your hour at room temperature is good because the clone you use works best there. But his clone works best in his conditions.

DR. LENKEI: No.

DR. BRAYLAN: We don't have a good standard.

DR. FISCHER: That's what I'm saying.

DR. LENKEI: If you stain for one hour the differences among clones will be less because you eliminate a lot of opportunity --

DR. FISCHER: That's not always true.

(inaudible)

DR. STETLER-STEVENS: We're not going to determine anything tonight that we're going to go with. Because we don't know what's best. What we know is that if you vary something and what we want to stop right here in the beginning is someone picking up a paper and saying gee I should quantitate CD20 and I'll just put this stuff in and I'm going to put the beads on. Because I have the beads I can do it. So what we have to have is a list of things that you have to optimize. I think we can come to agreement that you have to optimize certain things. After then it's the big trials that are going to determine the best method that works. You do it and you get a response that correlates to a specific antigen density. You report that.

But we don't want everybody to think is

okay I know that these factors are very important in being reproducible so therefore I have to look and say what is the temperature, what's the time, what's the antibody clone and how to go through and do all that so that when they are trying to reproduce it they think about what's important. That is something that we can be very helpful to the general community with. Then we can look at one antigen and try to determine the reproducibility. If we find out that each method is highly reproducible and gives you a different answer, that is still useful information.

DR. SCHWARTZ: To whom?

DR. STETLER-STEVENS: To the --

DR. FISCHER: My boss was the scientific director in one of the institutes at NIH just recently attended a meeting where they were talking about trying to get the FDA to recognize the presence of bio markers as indicators of disease. The fact that too many of the things aren't recognized because, lo and behold, there's no quantitation for them. They don't want to use them as indicators of a disease state because there isn't a quantitative method to give you a hard number and say that this is what it is. Because

everybody has their own opinion on what really is an indicator for that. You heard from Lance on the stuff he did with the CD38 because, God knows when I look at my CD38 on my B-cells on normal individuals, it's pretty dim. You get some of these disease states it's brighter than anything else out there.

PARTICIPANT: Mary Alice, the problem is we're trying to run -- What you are saying about coming up with a list of things that you do, that actually needs to be expanded. You need to come up with consensus of how you validate an assay. Maybe that's the end goal for this meeting.

DR. STETLER-STEVENS: What you have to validate and how you validate an assay. That would be the third action item. We need to come up with instructions as to how do you validate your assay.

DR. SCHWARTZ: That's later.

DR. STETLER-STEVENS: This is only sample preparation we talked about. We have not yet gone into compensation.

DR. MARTI: That's tomorrow.

DR. STETLER-STEVENS: Analyses, all these other things. And the beads versus cells. What marker are you going to use? We're just

talking about how you are going to stain the cell. Are you going to lyse them? What are you going to do? We'll never reach consensus in this group this year on exactly how we're going to do everything, but we can come up with consensus of what's important, how do you determine if you are doing it right, and we can look at some methods for one because it will be an instructive exercise for all of us. We'll learn something from it. Others will learn from the experience when you report it. Tomorrow we're going to have to talk about compensation in QC. We can't argue as much because we won't get through it. I think people are starting to go out the door. Do people want to stop now?

DR. MARTI: Is there a consensus that we can start at 8:00 a.m.? That's not too early. All those in favor of 8:00 a.m. signify by saying aye.

(Chorus of ayes.)

DR. MARTI: Opposed? Abstentions? Unanimous. It's 8:00 a.m. Off the record.

(Whereupon, the above-entitled matter was concluded at 5:44 p.m.)