Appendix 9
Minutes of VMT meeting

VMT meeting for Epidermal Sensitization Assay

Day 1: Wednesday, July 4, 2018

VMT: David Basketter, Chantra Eskes, Sebastian Hoffmann, David Lehmann, Tae Sung Kim, Masahiro Takeyoshi, Masaaki Miyazawa, Hideyuki Mizumachi, Hajime Kojima, Takao Ashikaga, Takashi Sozu
Observers: Representatives of participating labs

Kojima:	(Self-introductions) Today we will hear an explanation of the protocol, then the participating laboratories will present the results of the transferability study, and then we will discuss the study plan. Tomorrow, we will discuss chemical selection. Also, I would like the VMT to express an opinion about whether this test can be used as a standalone test or should be part of an IATA.
Mizumachi:	Presentation of test protocol (see PowerPoint presentation)
Eskes:	Is there is a specific time for determining a stable solubility?
Mizumachi:	No
Basketter:	I am wondering if not having a solvent control is problematic. How do we know what the effect of the vehicle is? But perhaps we shouldn't get into this too deeply at this point.
Hoffmann:	What is the preferred order of solvents? You test all three and use the one with the highest concentration?
Miyazawa:	Correct.
Hoffmann:	BADGE is not a well-defined substance, which might be problematic.
Basketter:	Yes, it will be interesting to see what happens when BADGE is used at the participating laboratories.
Basketter:	The Benzopyrene results might be dangerous because it suggests that all negative chemicals must be tested for 24 hours to ensure they do not give positive results.
Hoffman:	I would like to discuss testing of liquids.
Basketter:	I am concerned that there might be differences between application of test chemicals in a vehicle and neat test chemicals. We need to make reference to the effect of the vehicle. Also, we need to determine if there is a difference between neat concentrations and 50% concentrations of liquid test chemicals. For example, is there any potential for solid test chemicals that are negative at a 50% solution to be positive if they were to be tested at 100%.
Hoffmann:	I agree with David. If we could see an analysis without the 100% concentrations, we could get an idea if there is an issue that needs to be addressed here.
Basketter:	And we would know that the use of 100% concentration was available in special circumstances but not necessary on a regular basis.
Eskes:	When did you the LDH assay rather than the MTT assay?
Miyazawa:	There are some issues as shown on page 13 of the SOP. But LDH can be performed with just the supernatant rather than with a tissue sample, so it is more practicable than the MTT assay.
Basketter:	You said that if the solution had separated, you don't use it. But I would shake it and use it. I guess what I am really saying is that I don't understand what "stable dispersion" means. After 5 minutes? After 25 minutes? Do you need stability criterion, or does it really mean just a suitable dispersion? I don't think the VMT should be rewriting the SOP at this point, but I do think that this description needs to be clarified. Don't say "a few minutes." Describe specifically what you do and specify numbers.
Lehmann:	Given the controls and other things that are tested, can a lab technician typically perform a test of up to five concentrations?
Mizumachi:	Typically, we test four concentrations, so this is possible.
Eskes:	So, this isn't really a dose response, it's just yes or no.
Hoffmann:	And the more concentrations you test, the more likely you are to get false positives. And you said that for non-cytotoxic chemicals you do three concentrations?
Mizumachi:	At least three. And for cytotoxic chemicals, three to five concentrations.
Basketter:	What is going on in my mind is how other people will view this. They will see that the protocol calls for three to five concentrations and that your test results only have three concentrations. So, they will tell you to go back and do five concentrations, unless you provide a rationale for only having three. So, if you have a steep slope, you only need three concentrations to reach 80% viability. But if you have a shallow slope, you need five.

Hoffmann:	How relevant is the applicability domain and how can we determine if an unknown substance can be tested?
Basketter:	The situation with benzapyrene should not be used as a rationale to limit the applicability domain. Can you identify three other exclusive pro-haptens---chemicals that test negative because they require metabolism? This is good science, but it can be misunderstood by someone not sufficiently familiar with the field. If you are going to exclude a substance, like FITC, that binds to keratin, you need to define how it binds. I would think that most sensitizers bind to keratin, so there must be a different reason that it is not predicted correctly.
Hoffman:	So how do we explain this problem with FITC? It might be difficult to do in a timely fashion while we are doing chemical selection and finalizing the protocol.
Basketter:	We could look at the test data for allergens and use that as rationale for why FITC is problematic, rather than binding to keratin.
Hoffmann:	I think we have covered most of the main issues
Ashikaga:	We would like to confirm that we don't need to increase exposure time.
Hoffman:	I don't think we do. I think we may come back to other protocol points during the afternoon discussions.
	End of morning session
Eskes:	We will now have a presentation on transferability, and then we will talk about the study plan.
Miyazawa:	Presentation on technology transfer (See PowerPoint presentation)
Lehmann:	It's seem that ATF3 is the most difficult to measure correctly.
Miyazawa:	AOO might affect ATF3. Perhaps some technicians are working too deliberately, so the acetone dissipates while being dispensed, leading to an olive oil rich chemical solution.
Basketter:	It was interesting that propylgallate was only positive because of ATF3. The results at Lion show a big jump in the ATF3 values, so I don't think this was just because of the technique of applying the test chemical solution.
Lehmann:	Of the chemicals that were positive because of ATF3, how many would be positive with a different vehicle?
Hoffmann:	It would take a lot of data mining, but I think it would be good to establish historical values for these criteria.
Basketter:	So there is an issue with ATF3 and BADGE as the positive control. But otherwise the test seems to be functioning well. So perhaps BADGE is not the best positive control.
Hoffmann:	In addition to my other reservations about BADGE, it also exhibits declining dose response curve.
Basketter:	The transferability results are not so conclusive. All the labs have gotten it right once and gotten it wrong once. Despite this, I think the work was done carefully, so the main issue here is unlikely to be lab technique.
Hoffmann:	There are different ways to approach solving this issue. Maybe three out of four results, or perhaps the cut-off value for ATF3 could be lowered.
Eskes:	So, we do see that there is an issue with the use of BADGE. So what kind of solutions can we consider.
Hitoshi:	Perhaps we can give greater weight to the other three genes or perhaps we can consider changing the positive control.
Eskes:	You will need to look at the different possibilities. For example, change the positive control, reintroduce a second positive control, or change the criteria 3 genes instead of 4 genes, consider introducing an acceptance criteria for the standard deviation, etc.
Takeyoshi:	Does the Ct value of GAPDH impact the results?
	Of the 130 chemicals, we did not see too many that were influenced by the Ct value of GAPDH, although I can't show you the data right now.
Hoffmann:	What do you do if all concentrations are cytotoxic?
Miyazawa:	We must retest at lower concentrations.
Basketter:	If the third tests are all successful, then all three participating labs will have had two successes and one fail. In which case, is that sufficient to proceed to the validation? And even if it is good enough, how are we going to deal with all the issues we have identified concerning the use of BADGE as the positive control? I rather like Hitoshi's idea that if one gene were to randomly fail, it should be ignored.
Eskes:	These aren't issues that we can answer without looking more closely at the data, so perhaps we will need a teleconference prior to the next F2F meeting.
Basketter:	So, after we get the results to the third test and have had a chance to look at the data, we should have a teleconference.
Kojima:	I will coordinate a teleconference in mid-August or early September.
Basketter:	It must be up to the Lead Laboratory to decide whether or not technology transfer to another lab is successful, but it is up to the VMT to make a decision about whether or not we can proceed from Phase 0 to Phase 1.

Eskes:	How about IP?
Miyazaw:	We have the patent only in Japan. But if accepted as an OECD test guideline, we will release the patent rights.
Lehmann:	Is this model available outside of Japan?
Miyazawa:	They are planning to make the tissue available in the US.
Eskes:	What about the use of the test as a standalone?
Kojima:	The examination of Phase 1 will start in October or November.
Basketter:	There was a comparison with LLNA and human data.
Miyazawa:	We expect similar predictive capacity to LLNA.
Basketter:	Yes, good sensitivity, but poor specificity. What are the 11 chemicals that gave false positives?
Kojima:	For regulatory purposes, it is important to avoid false negatives.
	(break)
Hoffmann:	The EpiSenA results are based on the prediction model for LLNA? Maybe you want to develop a prediction model for human data.
Basketter:	If all of those substances are sensitizing in humans, it is interesting that you did get positive results for all of them, even if some are only weak sensitizers in human.
Eskes:	Do we need to answer this question now, before we see more data?
Kojima:	I hope that you will eventually agree that this can be proposed as a standalone test.
Eskes:	Are there any comments about the outline of the study plan? I was looking at the performance standards and I have some concerns about Plan B.
Kojima:	Although the plan calls for 80% concordance for acceptability criteria, we must discuss whether to use that figure or adopt a different one as well as how many test chemicals.
Hoffmann:	I think we also need to have a statistical justification for how many chemicals we test. We can look at earlier validations that had such a justification and follow that model. So, there are 10 chemicals for which we have between-laboratory reproducibility. We could for example specify 80% concordance for between-laboratory reproducibility, and slightly higher, 85%, for within-laboratory reproducibility. And do power calculations for the number of chemicals. If the numbers match what was done for the h-CLAT/DPRA validation study, we could adopt that. So the first thing we should do is check to see if that is possible.
Eskes:	We need to know this for tomorrow's chemical selection. Is that possible?
Hoffman:	We could select thirty chemicals tomorrow and then if we need less, we can pare them down.
Eskes:	My concern is whether that is enough for WLR. Is 10 enough or do we need 15?
Hoffman:	We had an incompatibility problem with h-CLAT that we don't have now, so that we increased the required minimum of 13 to 15.
Eskes:	So, 30 overall should be sufficient for BLR?
Kojima:	You suggested 85% is normal for WLR. So, for Phase 1 we will need 13 times 3 = 39 chemicals.
Basketter:	The number of chemicals is 30. Of these, which we will select tomorrow, 15 will be for Phase 1 WLR. That leaves 15 more that can be used for BLR.
Hoffman:	Right, but the IL8 Luc PRP criticized the low number of test chemicals, so let's try to avoid having too few chemicals.
Basketter:	I also want to avoid the situation where we don't quite reach our target and end up arguing about whether we are close enough. But one thing I want to know how long it takes to assay a test chemical.
Miyazawa:	About one and a half days, including incubation, to finish testing. Usually we can test six or seven chemicals in one month per technician if tests are done every week.
Eskes:	We will need a statistical evaluation before we can finalize the number of chemicals.
Hoffmann:	Regarding timelines, I think we don't need more than two weeks to come to a decision to continue with phase 1B.
	Technology transfer to finish by end August 2018 Phase 1A from October 1 to February 19, 2019, followed by a teleconference in March (WLR, 18 tests of 6 chemicals) Phase 1B from April to November (WLR, 27 tests of 9 chemicals) Second face-to-face meeting in January 2020 Phase 2 from February to June 2020 (BLR, 15 tests of 15 chemicals) Final face-to-face meeting in July or August 2020
Sozu:	What is the merit of breaking up Phase 1?
Eskes:	It's an opportunity to address any major issues that come up during the early testing.

Thursday, July 5, 2018	
VMT: David Basketter, Chantra Eskes, Sebastian Hoffmann, David Lehmann, Tae Sung Kim, Masahiro Takeyoshi, Masaaki Miyazawa, Hideyuki Mizumachi, Hajime Kojima, Takao Ashikaga Observers: Steven Venti (meeting minutes)	
	h-CLAT/DPRA WLR: Assuming 95% concordance, power calculation resulted in 13 chemicals, which was increased to 15 chemicals BLR: Assuming 90% concordance, power calculation resulted in 21 chemicals, which was increased to 24 chemicals EpiSensA proposed success criteria of 85% for WLR and 80% for BLR Phase 1A: 5 chemicals Phase 1B: 9 chemicals Phase 2: 13 chemicals BLR based on 27 chemicals
Eskes:	Please review selection of the positive control reagents.
Hoffmann:	We can't expect a prediction model based on LLNA will correlate with human data, so we should also establish a prediction model based on human data.
Basketter:	The 11 false positive chemicals are mainly what I called Category 5, which are weak sensitizers in humans. In your list, you have the human results as negative, but where did you get those human results? Two of those chemicals are Category 4, which means they can be considered sensitizers in humans. In which case, you would have 16 of 25 chemicals concordant with human results. In any case, please double check those human results. And we need to clarify if the prediction model has not been optimized against human data.
Kojima:	So, let's select 30 candidate chemicals, and then Takao will verify availability in the Japanese market.
Basketter:	We have agreed to select 30 candidate chemicals, of which we will use 26. Maybe this should be 27 so that it is divisible by three. We want to select 20 positive and 10 negative chemicals, based on both LLNA and human data. Of the 20 positive chemicals, ten are to be GHS (LLNA) 1A and ten are to be GHS 1B. The 10 negative chemicals are to be GHS No Category. Other considerations: Lipophilicity, solids vs. liquids, pre- or pro-haptens. Also, already tested or new compounds? The final selection will include 9 each of 1A, 1B, and non sensitizers, resulting in a total of 27.
Ashikaga:	All the chemicals listed in the performance standards (for ARE-Nrf2) are available in Japan.
	Substances show in italics are recommended for Phase 1A. **Non-sensitizers** **CASRN** Dextran 3371-50-4 Diethyl toluamide 134-62-3 (candidate for exclusion) Triethanolamine 102-71-6 Tween 80 9005-65-6 Phenol 108-95-2 *Diethylphthalate* 84-66-2 (*recommended for Phase 1A)*

	Hexane 110-54-3 Propyl paraben 94-13-3 Para-aminobenzoic acid 150-13-0 *Sodium lauryl sulphate* 151-21-3 (-4 was positive in LLNA) (*recommended for Phase 1A)* Phenoxyethanol 122-99-6 (candidate to replace dextran) **1A sensitizers** *Glyoxal* 107-22-7 (*recommended for Phase 1A)* Tetrachlorosalicylanilide 1154-59-2 Dinitrochlorobenzene 97-00-7 (candidate for exclusion) Potassium dichromate 7778-50-9 *Lauryl gallate* 1166-52-5 (*recommended for Phase 1A)* Formaldehyde 50-00-0 Glutaraldehyde 111-30-8 Methyl heptine carbonate 111-12-6 Isoeugenol 97-54-1 Para-phenylene diamine 106-50-3 **1B sensitizers** Ylang ylang 8006-81-3 (for example) *Benzisothiazolinone* 2634-33-5 (*recommended for Phase 1A)* Ethyl acrylate 140-88-5 Phenyl benzoate 93-99-2 Abietic acid 514-10-3 Mercaptobenzothiazole 149-30-4 Amylcinnanic aldehyde 122-40-7 Lilial 80-54-6 (candidate for exclusion) Methylmethacrylate 80-62-6 Farnesol 4602-84-6

Teleconference for Epidermal Sensitization Assay

September 4, 2019

VMT: David Basketter, Chantra Eskes, Sebastian Hoffmann, David Lehmann, Masahiro Takeyoshi, Masaaki Miyazawa, Hideyuki Mizumachi, Hajime Kojima, Takao Ashikaga, Takashi Sozu, Tae Sung Kim
Observers:, Steve Venti (meeting minutes)

Kojima:	Today's agenda is as follows: 1. Welcome and approve draft agenda 2. Report on Phase 1A 3. Discussion of additional data using the revised protocol 4. Discussion of size of Phase 1B 5. Other business This draft agenda was approved by all.
Sozu:	(Presentation of the Statistical Analysis Report for Phase 1A)
Kojima:	Chemicals 1 and 4 have all concordant results, but 2, 3, and 5 do not. So we are concerned, because these results do not achieve the target criteria.
Basketter:	11 of the 15 combinations are fairly consistent, 4 of 15 are not. However, the predictions are incorrect. Unless there is some other information, there is a problem here that the Lead Laboratory has to solve to achieve the right predictions.
Kojima:	We are considering changing the protocol. And will report on that.
Miyazawa:	We will present a minor modification of the protocol, after which we can discuss the situation.
Mizumachi:	(Presentation of additional study using a modification to the protocol)
Basketter:	These improvements to the protocol are very good. But I still have a concern about the quality of the predictions for chemicals 2, 4, and 5. The predictions are consistent but incorrect.
Hoffman:	What does the Lead Lab have to say about this problem?
Miyazawa:	We don't know why chemical 2 results are incorrect.
Eskes:	Have these chemicals been tested by other alternative methods?
Mizumachi:	Yes, positive results I think.
Basketter:	What about 4 and 5?
Miyazawa:	There are some case reports of positive results for chemical 5.
Basketter:	The table on page 36 indicates that we need to do more work before we can move to validation.
Miyazawa:	This is the summary of EpiSensA results compared with other alternative tests. We feel that, even though the chemicals used in Phase 1A are a problem, this table shows good predictivity across a wide range of chemicals.
Basketter:	OK, that is a good response. In terms of predictive reproducibility, we need to have consistent prediction of a negative chemical. Other people might not agree, but that is my opinion.
Takeyoshi:	Yes, I agree with David.
Takao	I also agree.
Eskes:	I agree as well.
Basketter:	Chantra, do you remember any other times the results showed a similar situation with good reproducibility but poor predictivity.
Eskes:	Yes, and it results in a lot of discussion about how to explain the situation to the OECD, etc.
Basketter:	I think we on the VMT need to anticipate how best to handle this situation moving forward.
Kojima:	The participating laboratories are participating in this meeting, so we will discuss chemical selection afterwards.

	Last July, we determined the size of the study and said we would use 26 or 27 chemicals, then we selected 30 chemicals. We are now dealing with a limited budget and other issues, but I propose that we select 9 to 5 chemicals for Phase 1B.
Basketter:	If nine chemicals select, maybe four of 9 should be negative, and five should be positive: two 1A and three 1B.
Kojima:	That is a good balance. It is OK.
Miyazawa:	We would like to decrease the number of chemicals for Phase 1B. It took one year to test five chemicals for Phase 1A, so we would like to use fewer than nine chemicals for Phase 1B.
Hoffmann:	Our recommendation here was, as I recall, similar to h-CLAT.
Basketter:	So if we have five chemicals for Phase 1B, we would have to increase the number of chemicals for Phase 2, perhaps to 16 or so.
Hoffmann:	We set it up for Phases 1A and 1B to inform WLR, and then Phase 2 to inform BLR.
Basketter:	So, if we reduce Phase 1B, we will need to add back in to Phase 2. I think that we are happy with reproducibility, it is the lack of specificity that is an issue.
Hoffmann:	I am not all that confident about the WLR, because they had to make adjustments to get it. And if we have to adjust again for BLR, there could be problems.
Basketter:	I think we should stick to the plan [we agreed to last July].
Kojima:	The problem is that it took a year to do five chemicals, and we have limited time and budget. So I would like to decrease the number of chemicals in 1B.
Eskes:	How about having subphases for Phase 1B?
Basketter:	I am not comfortable with being pressured to do things because of restrictions on time and money. That seems like too much of a compromise.
Eskes:	Which is why taking an intermediate step would give us the opportunity to discuss how to proceed. We can have a Phase 1B and Phase 1C.
Basketter:	Can we do Phase 1B and Phase 1C, each with five chemicals? And then go to Phase 2. Every shortcut we take early on with just create problems at ECVAM or OECD level. The VMT has to be able to write a report showing that we are confident in our results.
Hoffmann:	I agree with David. Every shortcut now creates problems later, and I wonder why we were unable to keep to the schedule we agreed to last July. What happened to that schedule?
Kojima:	Phase 1A was supposed to be finished April. But the data was not good, and the protocol was revised, which necessitated additional testing.
Basketter:	So Phase 1B should go according to schedule?
Kojima:	Yes, it will take about five months.
Eskes:	As I remember, the participating labs were concerned even last year that they might not be able to keep the schedule.
Basketter:	Let's call it 1B and 1C, with 1C "to be discussed." If possible, it would be good to have a face-to-face meeting.
Kojima:	So, Phase 1B will test 5 chemicals, Phase 1C will test 5 chemicals, and Phase 2 will test 12 chemicals. Is that acceptable?
Miyazawa:	We might need to discuss human resources and budgeting with the participating laboratories.
Basketter:	I realize it will be challenging, but these numbers are necessary to validate the WLR and BLR. I think that reducing these numbers will very quickly make things unacceptable to the OECD.
Hoffmann:	There is a precedent set by previous validations [that we too must follow]. If the burden is too great, perhaps it will be necessary to add more labs.
Eskes:	What was the main reason for the delay? Was it because of the participating labs or was it because of the changes to the protocol?

Miyazawa:	It takes a month to prepare 3D models. So any problem creates a one month wait for new 3D models.
Basketter:	I understand these issues, but that shouldn't really affect how the VMT manages the process. I think that h-CLAT and DPRA really represent the minimum of what is acceptable for a validation, and any reduction past that is difficult to rationalize.
Kojima:	We would like to select five chemicals for Phase 1B and supply them to the participating labs by next month.
Basketter:	Maybe we can ask Drs. Miyazawa and Mizumachi to suggest five candidate chemicals, and we can comment on their selection. Three of 5 should be negative, and two should be positive: one 1A and one 1B.

Teleconference for VMT web meeting on EpiSensA for skin sensitization

March 12, 2020

VMT: Hofmann S., Basketter D., Lehmann D., Eskes C., Kim TS., Takeyoshi M., Miyazawa M., Mizumachi H., Sozu T., Kojima H., Ashikaga T., Venti S.
Participating Laboratories: Sakuma M. Imai N, Shibata M (Kose), Watanabe S., Ueno J. (Lion), Kojima K. Watanabe M. (FDSC)
Observers: Steve Venti (meeting minutes)

Kojima:	Thank you all for joining this meeting and cooperating with JaCVAM activities.
Mizumachi and Miyazawa:	Report of validation results for phase 1A and protocol modification (See presentation.)
Eskes:	Were there any change for chemicals No. 1, 2, and 4?
Miyazawa:	There was no change.
Kojima:	QC report for phase 1B (See presentation.) I am concerned about No. 31.
Ueno:	ATF gene expression did not meet the acceptance criteria for No. 31. The manufacturing lot for the clotrimazole was not changed, so we suspect that the chemicals did not dissolve completely.
Basketter:	This is a very straightforward explanation, and it's too bad this can't be retested.
Kojima:	So, we will have to delete No. 31 from the validation results.
Eskes:	Do you mean the other chemicals, too?
Kojima:	We retested the chemicals. We recommend using the results for No. 32.
Sozu:	Here is a statistical analysis for Phase 1B. (See presentation.)
Hoffmann:	Were these results obtained with the new protocol?
Sozu:	Yes.
Hoffmann:	So the revisions produced an improvement?
Eskes:	"New protocol" means the protocol we agreed on last September?
Sozu:	Yes.
Ashikaga:	I am wondering if we might not be able to eliminate the IL-8 measurement.
Miyazawa:	We should not judge that from Phase I-B results because there are only two positive chemicals. On the other hand, the large dataset containing over 130 chemicals have been developed, and we confirmed that some sensitizers containing strong one only yields IL-8 expression. Therefore, IL-8 is necessary.
Kojima:	So, we can accept this data and discuss the next revision of the protocol.
Mizumachi and Miyazawa:	Proposed minor revision of protocol (See presentation.)
Eskes:	Have you used the new RNA extraction with the EpiSensA protocol?
Miyazawa:	I haven't checked the positive control, but the gene expression of the three test chemicals using the Maxwell method gave results that were very similar to those for TRIzol method.
Basketter:	There seems to be little difference for these three sensitizers. Will you use the Maxwell method in the next phase?
Miyazawa:	No. The rest of the validation is always being performed with the TRIzol method.
Lehmann:	So, the Maxwell will be offered as an option, but the rest of the validation will be performed only with the TRIzol?
Miyazawa:	Yes.
Basketter:	This is a good development that will provide additional data in future, but I think that it is importation to continue to use the TRIzol for the rest of the validation.

Kojima:	So the new protocol will include the Maxwell as an option?
Miyazawa:	Yes.
Hoffmann:	If it is not going to be used in the validation, then it isn't really relevant to our discussions. Is there a reason to add it now?
Miyazawa:	We have to make an SOP and would like to add this method as an option in the SOP.
Basketter:	I understand why you want to add it, but I think perhaps it should be in an appendix. If the validation is being performed with TRIzol, then the protocol and the SOP should include only TRIzol. Also, an additional step is required to show the equivalence of the Maxwell method, and that has yet to be performed. So, that data should be included in an appendix, too.
Kojima:	If possible, I would like to discuss the Maxwell method again at a face-to-face meeting after Phase 1C and before Phase 2. But Phase 1C should be performed with the current protocol.
Miyazawa:	Well, what about the other proposed revisions to the descriptions?
Basketter:	I think the revisions to the descriptions are entirely reasonable.
Lehmann:	Yes, and the pictures are helpful, as well.
Ashikaga:	I don't think the measurement should be performed immediately after stopping the reaction. I don't think that HCl stops the reaction perfectly. Why do you propose this?
Miyazawa:	One of the laboratories requested that we clarify the timing of the measurement, which we did based on instructions from the supplier.
Ashikaga:	OK.
Kojima:	Does everyone agree with these proposed minor revisions to the descriptions?
All:	Agreed.
Kojima:	We will now select five more chemicals for testing in Phase 1C and we will distribute them to the labs by the end of March, so Phase 1C will start in April and I hope to be finished by August. I hope to have a face-to-face meeting in September. Can the laboratories agree to this schedule?
Three Labs:	We agree.
Kojima:	I would like to start Phase 2 in October or November and complete the validation study next year.
Basketter:	That sounds good.
Sozu:	I would like Phase 1-C testing to be finished by the end of July, so that I can prepare the report in August.
Kojima:	What do the labs think?
Sakuma:	We will try.
Ueno:	It will depend on the chemicals, but we will try.
Watanabe:	It will be difficult.
Kojima:	I hope it will be possible to finish by the end of July, but I think the middle of August will be acceptable.
Basketter:	If the quality of results are as good as what we saw today, the statistical analysis will be very easy.
Kojima:	So, I would like to ask the participating laboratories to leave the meeting, so that the VMT can discuss the chemical selection.

The 4th VMT web meeting on Epidermal Sensitization Assay for skin sensitization

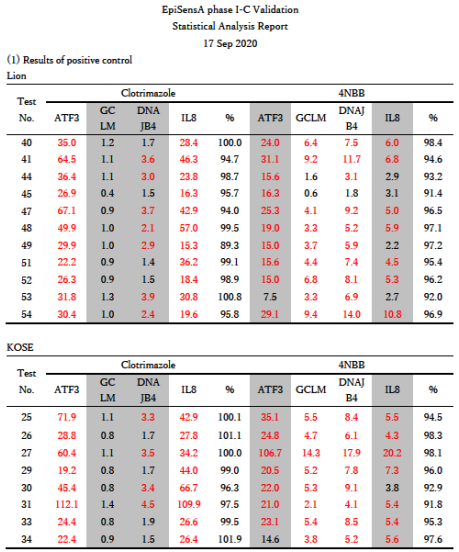
September 23 and 24, 2020

Overview on the meeting on September 23rd

- Dr. Sozu, a biostatistician, reported the outcome of phase I-C and mentioned that the fold induction of positive control at some runs at set 2 of LION (Lab.A) did not meet the criteria.
- The within-laboratory reproducibility of Lab.A, Lab.B and Lab.C was each 100%, 80% and 80%.
- All records were confirmed by JaCVAM.
- LION‘s failures were caused by the cross-contamination effect of chemical No.13, inducing marked positive responses and may be related to test compound volatility and potency. . The discordant results (GCLM, DNAJB4) of chemical No.11 at Lab.B and C were caused by the same reason.
- The other labs re-tested some runs due to technical errors or strong cytotoxicity at tested concentrations.
- LION‘s failures were caused by the cross-contamination effect of chemical No.13. The discordant results (GCLM, DNAJB4) of chemical No.11 at Lab.B and C were caused by the same reason.
- The borderline positive result (IL-8) of chemical No.11 at set 3 of Lab.B was likely caused due to concentrations around 80% viability, but the overall results of Chem. No.11 were negative.
- The cross-contamination effect at Phase 1-A and 1-B was unlikely caused.
- To avoid this trouble, the following addition of the revised protocol was accepted.

To avoid cross-contamination by volatile compounds, the tissue units which are used for liquid test chemicals should be separated from other test chemicals and controls into individual 24-well plates.

- The VMT members agreed to complete phase I-C.



EpiSensA phase I-C Validation
Statistical Analysis Report
17 Sep 2020

(1) Results of positive control

Lion

Test No.	Clotrimazole					4NBB				
	ATF3	GCLM	DNAJB4	IL8	%	ATF3	GCLM	DNAJB4	IL8	%
40	35.0	1.2	1.7	28.4	100.0	24.0	6.4	7.5	6.0	98.4
41	64.5	1.1	3.6	46.3	94.7	31.1	9.2	11.7	6.8	94.6
44	36.4	1.1	3.0	23.8	98.7	15.6	1.6	3.1	2.9	93.2
45	26.9	0.4	1.5	16.3	95.7	16.3	0.6	1.8	3.1	91.4
47	67.1	0.9	3.7	42.9	94.0	25.3	4.1	9.2	5.0	96.5
48	49.9	1.0	2.1	57.0	99.5	19.0	3.3	5.2	5.9	97.1
49	29.9	1.0	2.9	15.3	89.3	15.0	3.7	5.9	2.2	97.2
51	22.2	0.9	1.4	36.2	99.1	15.6	4.4	7.4	4.5	95.4
52	26.3	0.9	1.5	18.4	98.9	15.0	6.8	8.1	5.3	96.2
53	31.8	1.3	3.9	30.8	100.8	7.5	3.3	6.9	2.7	92.0
54	30.4	1.0	2.4	19.6	95.8	29.1	9.4	14.0	10.8	96.9

KOSE

Test No.	Clotrimazole					4NBB				
	ATF3	GCLM	DNAJB4	IL8	%	ATF3	GCLM	DNAJB4	IL8	%
25	71.9	1.1	3.3	42.9	100.1	35.1	5.5	8.4	5.5	94.5
26	28.8	0.8	1.7	27.8	101.1	24.8	4.7	6.1	4.3	98.3
27	60.4	1.1	3.5	34.2	100.0	106.7	14.3	17.9	20.2	98.1
29	19.2	0.8	1.7	44.0	99.0	20.5	5.2	7.8	7.3	96.0
30	45.4	0.8	3.4	66.7	96.3	22.0	5.3	9.1	3.8	92.9
31	112.1	1.4	4.5	109.9	97.5	21.0	2.1	4.1	5.4	91.8
33	24.4	0.8	1.9	26.6	99.5	23.1	5.4	8.5	5.4	95.3
34	22.4	0.9	1.5	26.4	101.9	14.6	3.8	5.2	5.6	97.6

1

Flow chart to verify the effect of cross-contamination

i) Were test chemicals or vehicle controls put in the same plate with Chem.No.13?

No

YES

ii) Verify if over/under-evaluation was caused by cross-contamination.

Did the Imax value fall outside of the variation?

No → Cross-contamination of Chem. No.13 did not affect the P/N judgment and WLR.

YES

iii) Verify if change of P/N judgment was caused by cross-contamination.

Were the Imax values of *ATF3* and *IL-8* less than the cut-off value?

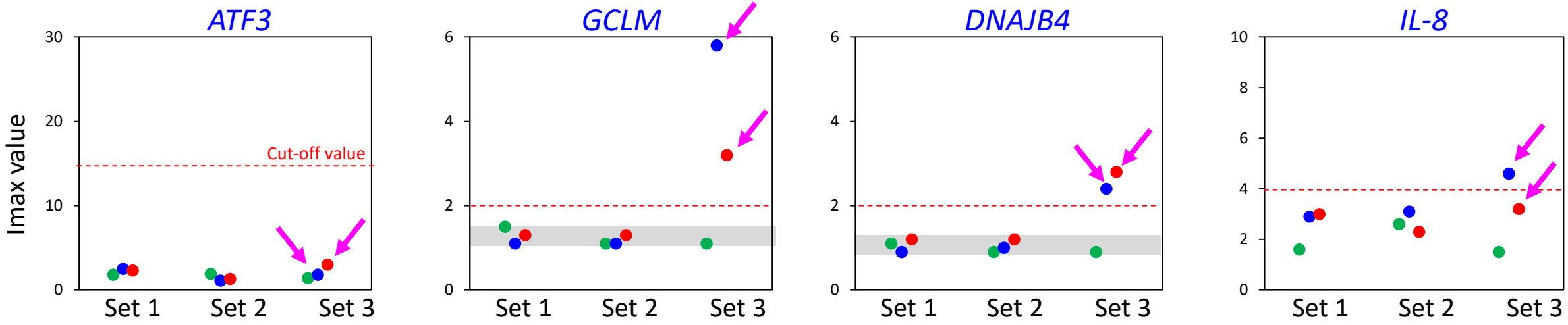
No

YES

Cross-contamination of volatile Chem. No.13 likely affected the P/N judgment and WLR.

Chemical No.11

● Lab.A ● Lab.B ● Lab.C : Test chemical was put in the same plate : Vehicle cont. was put in the same plate



i) Were test chemicals or vehicle controls put in the same plate with Chem.No.13?

● Lab.A : NO ● Lab.B : Set 3 ● Lab.C : Set 3

ii) Did the Imax value fall outside of the variation?

● Lab.B : YES ● Lab.C : YES

iii) Were the Imax values of *ATF3* and *IL-8* less than the cut-off value?

● Lab.B : NO (*IL-8* Posi) ● Lab.C : **YES**

Overview on the meeting on September 24th

1. The within-laboratory reproducibility of Lab.A, Lab.B and Lab.C was each 93.3%, 93.3% and 86.7% in phase I. The proportion of concordance within-laboratory reproducibility met more than 85% as acceptance criteria of phase I .
2. The proportion of concordance between-laboratory reproducibility was 86.7% in phase I and there is more than 80% as acceptance criteria at this time.
3. Predictivity in all labs. was similar with that of lead lab.
4. The coded Number was broken(see page 7) and the volatile chemical No.13 was clarified to be Methyl heptine carbonate, a clear LLNA and human Cat. 1A sensitizer.
5. The VMT discussed the chemical selection for phase II chaired by Dr. D. Basketter

1) The VMT agreed twelve as the number of tested chemicals.
2) Based on DASS database, the chemicals were selected.
3) The information of LogKow is included in table.
4) The candidate chemicals were selected based on KAO's draft proposal.
5) By e-mail, we continue to discuss the candidate ones and they will be fixed by the end of October 2nd.

6. Future plan (see page 9) was proposed from Kojima and all members agreed.

Summary of Phase I (predictivity)

Chemical No.	GHS Cat.	Lab. A			Lab. B			Lab. C		
		set 1	set 2	set 3	set 1	set 2	set 3	set 1	set 2	set 3
1	1A	P	P	P	P	P	P	P	P	P
2	1A	N	N	N	N	N	N	N	P	N
3	1B	N/P	N/N	N/N	P	P	N/P	P	P	N/P
4	NC	P	P	P	P	P	P	P	P	P
5	NC	P	P	P	P	N/P	P	P	P	P
6	NC	N	N	N	N	N	N	N	N	N
7	NC	N	N	N	N	N	N	N	N	N
8	NC	N	N	N	N	N	N	N	N	N
9	1A	P	P	P	P	P	P	P	P	P
10	1B	P	P	P	P	P	P	P	P	P
11	NC	N	N	N	N	N	P	N	N	P
12	1A	P	P	P	P	P	P	P	P	P
13	1A	P	P	P	P	P	P	P	P	P
14	1B	P	P	P	P	P	P	P	P	P
15	1B	P	P	P	P	P	P	P	P	P

No.13 might affect the P/N judgment

✓ Predictivity for 15 chemicals;

(discordant results was judged by median classification*)

	Phase I chemicals				Dataset
	Lab.A	Lab.B	Lab.C	Kao	Kao
n	15				136
Sens.	78%	89%	89%	89%	88%
Spec.	67%	67%	67%	67%	66%
Accu.	73%	80%	80%	80%	82%

Predictivity at participating Lab. was comparable to Kao data and the dataset of EpiSensA.

	h-CLAT validation*				Dataset
	Kao	Shiseido	Bioassay	ECVAM	OECD TG
n	24				142
Sens.	75%	88%	75%	88%	93%
Spec.	63%	75%	75%	50%	66%
Accu.	71%	83%	83%	75%	85%

That was also comparable to h-CLAT validation results and the dataset adopted to OECD TG442E.

*: h-CLAT validation study report

EpiSensA_P1C Coded Chemicals

作成日：2020.9.17

No.	Chemical name	CAS No.	LabA LION			LabB KOSE			LabC FDSC			Remark	Storage	Physicality	Supplier	Lot	Product code
			set1	set2	set3	set1	set2	set3	set1	set2	set3						
11	Lactic acid	50-21-5	ESC725	ESC821	ESC923	ESC123	ESC225	ESC324	ESC421	ESC524	ESC622		R	Liquid	SIGMA	MKBW2171V	L6661
12	p-Phenylenediamine	106-50-3	ESC721	ESC824	ESC924	ESC121	ESC224	ESC322	ESC422	ESC521	ESC625	H	R	Solid	Sigma-Aldrich	WXBC8316V	P6001
13	Methyl heptine carbonate	111-12-6	ESC723	ESC822	ESC925	ESC125	ESC222	ESC325	ESC424	ESC525	ESC621	D	R	Liquid	Sigma-Aldrich	MKBR4298V	277509
14	Abietic acid	514-10-3	ESC724	ESC825	ESC921	ESC124	ESC223	ESC323	ESC423	ESC523	ESC624		C	Solid	Sigma-Aldrich	BCBR5079V	00010
15	Famesol	4602-84-0	ESC722	ESC823	ESC922	ESC122	ESC221	ESC321	ESC425	ESC522	ESC623	D	R	Liquid	Aldrich	MKCJ1135	F 203

D=Deleterious Substance

H=Dangerous Substance

R=Room Temperature

C=Refrigeration storage

Candidate chemicals of phase II

-: not described

n.a. : not available

	Chemical name	CAS No.	LogKow	Solid /liquid	LLNA		Human		OECD DASS DB 2020-06-17		EpiSensA		DPRA	Keratino Sens	h-CLAT
					EC3 (%)	ref.	Category	ref.	LLNA	Human	Judge	Comments			
NC	propylene glycol	57-55-6	-0.78	L	NS	f	5	c	NC	NC	N	Borderline negative	N	N	N
	Octanoic acid	124-07-2	3.03	L	NS	f	6	c	NC	NC/1B	P	Borderline positive	N	N	P
	Isopropanol	67-63-0	0.28	L	NS	f	5	c	NC	n.a.	N	Borderline negative	N	N	N
	Acetanisole	100-06-1	1.75	S	NS	a	N	a	NC	NC/1B	N	Borderline negative	N	P	N
1A	Tetrachlorosalicylanilide	1154-59-2	5.87	S	0.04	f	1	c	1A	1A	P	Clear positive	P	P	P
	Isoeugenol	97-54-1	2.65	L	1.35	f	2	c	1A	1	P	Clear positive	P	P	N
	2-Aminophenol	95-55-6	0.6	S	0.45	f	2	c	1A	n.a.	P	Clear positive	P	P	P
	Glutaraldehyde	111-30-8	-0.18	L	0.09	f	2	c	1A	1	P	Borderline positive	P	P	P
1B	Lilial	80-54-6	4.36	L	11.35	f	4	c	1B	1B	P	Clear positive	P	N	P
	Methyl methacrylate	80-62-6	1.28	L	75	f	4	c	1B	NC/1B	P	Clear positive	P	P/N	P
	Amyl cinnamic aldehyde	122-40-7	4.33	L	11.2	f	4	c	1B	NC/1B	P	Clear positive	N	P	P
	Imidazolidinyl urea	39236-46-9	-8.28	S	24	f	3	c	1B	1B	P	Borderline positive	P	P	P

References

a: Urbisch, et al., Regul Toxicol Pharmacol., 2015.

b: Kleinstreuer, et al., Crit Rev Toxicol., 2018.

c: Basketter, et al., Dermatitis, 2014

d: Jaworska, et al., Arch Toxicol., 2015

e: h-CLAT validation study report

f: Hoffmann et al., 2018

g: Newly tested by Kao

Future plan

October, 2020	Distribute the coded chemicals for phase II
November, 2020	Start the experiment for phase II
April, 2021	End for phase II
May or June, 2021	VMT meeting (if possible, F2F meeting in Tokyo)

Minutes
The VMT web meeting on Epidermal Sensitization Assay for skin sensitization

Date: July 13, 2021, 9 pm-11 pm (Japan), 8am-10am (US East) and 2pm-4pm (CE)

Participants: Basketter D., Lehmann D., Eskes C., Takeyoshi M., Miyazawa M., Mizumachi H., Sozu T., Kojima H., Ashikaga T.
Participated laboratory: Sakuma M. Mizuno M, Shibata M (Kose), Watanabe S., Ueno J. (Lion), Watanabe M. (FDSC)

1. Welcome address and approve of draft Agenda

Hajime welcomed the all members and asked them to approve the draft agenda for the meeting. All the members agreed.

2. Results of phase II by biostatisticians

Takashi introduced the statistical results of phase II as a biostatistician. The results are available at the attached file.

Regarding some data have not yet met positive criteria on ATF3, between laboratory reproducibility with the accepted data is 90 % (18/20) and met the acceptance criteria of prediction model.

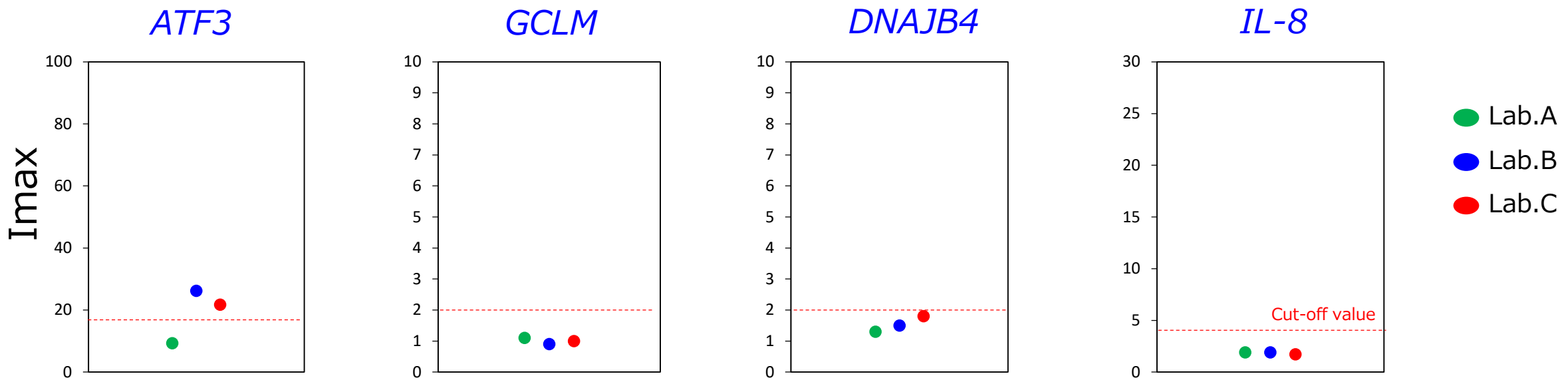
3. Overview from Lead Lab.

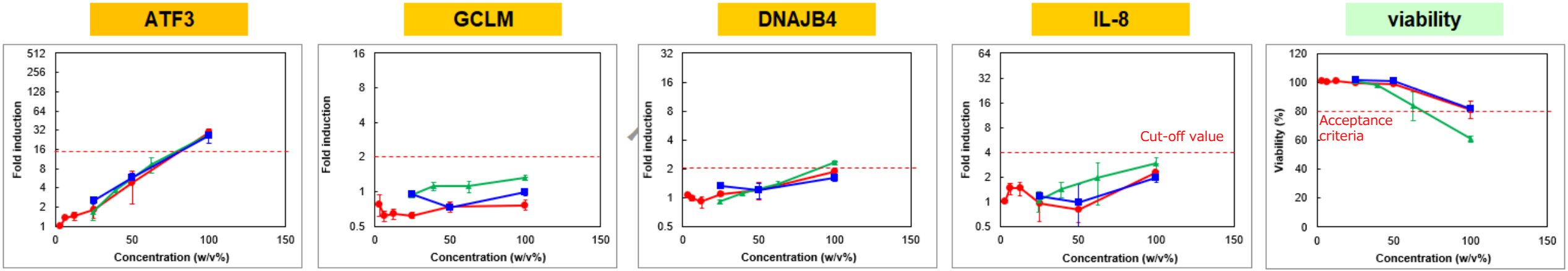
1) Consideration about discordant results

- In the Phase II, 10/12 chemicals showed concordant results among three laboratories.
- Discordant results were confirmed at Chemical No.1 and No.2.
- In terms of Chemical No.1, Lab.A judged as negative but Lab.B and C judged as positive. Dose response of three labs were very similar at *ATF3*, and the fold induction exceeded the cut-off value at 100w/v% concentration. However, viability at 100w/v% concentration showed less than 80% at Lab.A, and the result at this concentration was discarded. For this reason, discordant result was confirmed at Chemical No.1.
- In terms of Chemical No.2, Lab.B judged as negative but Lab.A and C judged as positive. Imax of *IL-8* at Lab.B was very close to cut-off value. Therefore, Chemical No.2 might be difficult to obtain concordant result.
- Chemical No.1 and 2 were tested 3 and 5 times by Kao, respectively. Chemical No.1 was judged as positive at 1/3 runs, and Chemical No.2 was also done at 1/5 runs.

Chemical No.	Lab.A	Lab.B	Lab.C
1	N	P	P
2	P	N	P
3	N	N	N
4	P	P	P
5	P	P	P
6	P	P	P
7	P	P	P
8	P	P	P
9	P	P	P
10	P	P	P
11	P	P	P
12	P	P	P

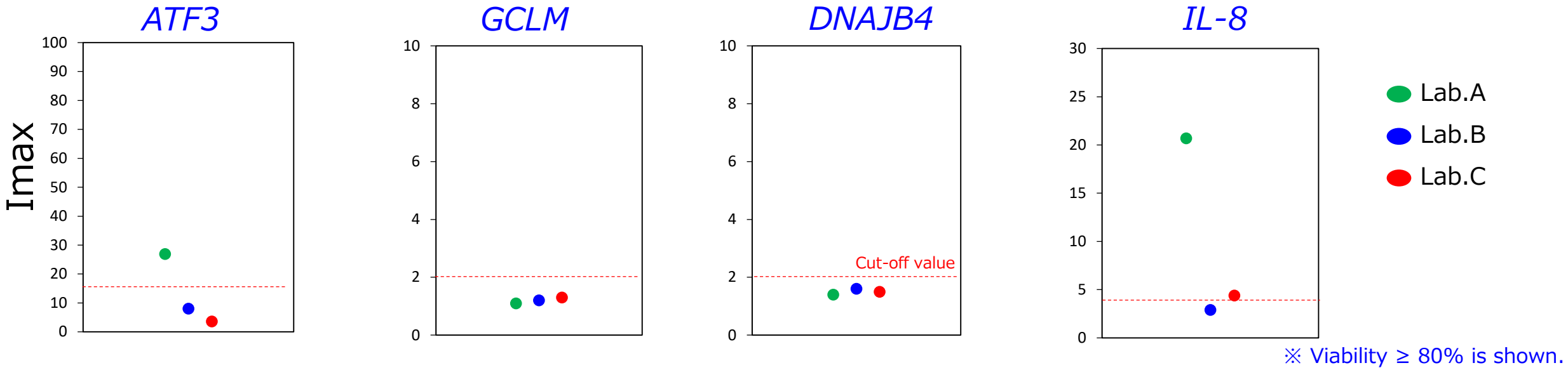
Chemical No.1



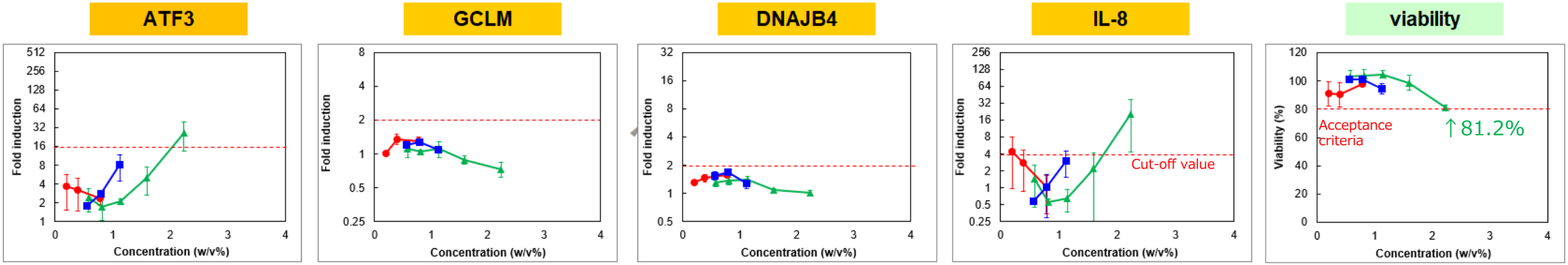


- ✓ Dose response of three labs were very similar at *ATF3*.
- ✓ Viabilities at 100w/v% were around criteria, and showed <80% at Lab.A.

Chemical No.2



※ Viability ≥ 80% is shown.



- ✓ Imax of *IL-8* at Lab.B was very close to cut-off value.
- ✓ Lab.A showed greater *IL-8* Imax because acceptable conc. was higher than other labs.

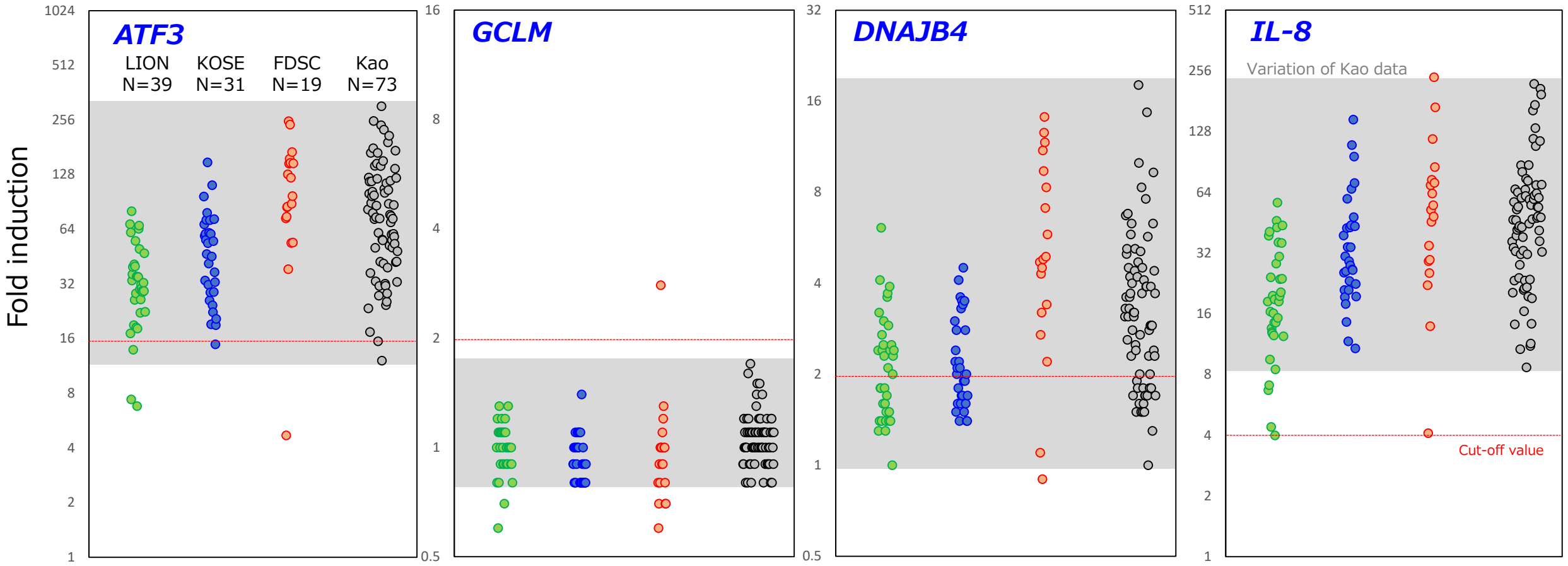
3. Overview from Lead Lab.

2) Consideration about positive control

- *ATF3* of clotrimazole did not meet the acceptance criteria (15-fold) in 3/9 runs at LION and 1/8 run at KOSE.
- In terms of LION‘s failures, the RhEs with a high concentration of test chemical was re-used as killed control, and cross-contamination of the test chemical to AOO vehicle control might be occurred.
- *ATF3* gene expression increased at cross-contaminated AOO control, and 0.78% clotrimazole was under-evaluated, which could be the reason for LION’s failures.
- To avoid the cross-contamination, SOP of EpiSensA will be revised.
- Fold induction of KOSE at failed run (14.9-fold) was very close to cut-off value (15-fold), and *ATF3* fold inductions of KOSE fell within the variation of Kao historical data. In addition, there might not be a significant difference between KOSE and Kao about frequency of failed run.
- Almost fold inductions of all marker genes obtained by participating laboratories fell within the variation of Kao historical data.

		0.78% Clotrimazole				
Laboratory	Run No.	*ATF3*	*GCLM*	*DNAJB4*	*IL-8*	Viability (%)
LION	59	**29.2**	1.0	1.4	**23.8**	104.7
	61	10.3	2.9	4.1	**9.0**	99.3
	62	**28.9**	1.0	2.5	**20.5**	103.7
	63	4.4	0.7	1.6	**5.0**	100.6
	64	**32.5**	0.9	1.0	**35.9**	100.6
	65	**29.3**	1.0	2.0	**23.9**	99.9
	67	**47.3**	0.9	2.3	**43.9**	99.5
	69	11.3	0.9	3.3	**14.5**	96.4
	70	**22.5**	0.8	2.4	**12.4**	97.5
KOSE	36	**55.2**	1.0	3.5	**146.9**	99.2
	37	**28.9**	0.8	1.9	**48.3**	97.6
	38	**72.8**	0.9	2.8	**96.4**	100.8
	39	**37.2**	0.8	1.6	**71.2**	100.7
	41	**32.8**	0.9	2.0	**43.5**	100.4
	42	14.9	0.8	1.4	**10.8**	98.7
	43	**19.0**	0.8	1.4	**22.5**	99.1
	44	**20.6**	0.9	1.7	**19.5**	98.3
FDSC	2	**170.4**	1.0	8.3	**238.5**	99.0
	3	**97.5**	0.8	3.4	**71.4**	98.7
	4	**54.2**	0.7	2.2	**85.4**	98.2
	6	**147.6**	0.7	5.8	**168.9**	98.7

Comparison of gene expression at 0.78% clotrimazole



Almost data of three labs. fell within the variation of Kao historical data.

3. Overview from Lead Lab.

3) Overall result of BLR

- 24 out of the 27 chemicals showed concordant results among three labs. Therefore, BLR was 88.9% and met the success criteria of 80%.
- The VMT members agreed to complete the Phase II. Hajime decoded the chemical list for phase II.

EpiSensA Phase2

No.	Chemical Name	CAS No.	LabE LION	LabF KOSE	LabG FDSC
1	propylene glycol	57-55-6	ESE141	ESF238	ESG342
2	Acetylanisole	100-06-1	ESE134	ESF239	ESG331
3	Benzylbutylphthlate	85-68-7	ESE131	ESF236	ESG341
4	1-Iodohexane	638-45-9	ESE142	ESF242	ESG340
5	Tetrachlorosalicylanilide	1154-59-2	ESE133	ESF241	ESG338
6	Isoeugenol	97-54-1	ESE139	ESF237	ESG332
7	2-Aminophenol	95-55-6	ESE138	ESF233	ESG337
8	50%Glutaraldehyde	111-30-8	ESE137	ESF240	ESG335
9	Lilial	80-54-6	ESE132	ESF234	ESG333
10	Methyl methacrylate	80-62-6	ESE136	ESF235	ESG334
11	Amyl cinnamic aldehyde	122-40-7	ESE135	ESF232	ESG336
12	Imidazolidinylurea	39236-46-9	ESE140	ESF231	ESG339

4) Overall predictivity

- Validation chemicals contain 10 of “not categorized” chemicals, 9 of “category 1A”, and 8 of “category 1B”.
- Sensitivities and accuracies of validation results at three labs. were comparable to those of Kao dataset.
- Regarding specificity, there might be slight differences between validation results and Kao dataset, but the differences derived from only one or two false positive at validation chemicals. In addition, it is likely that specificity of EpiSensA fell within other test methods.

	Validation			Dataset
	LION	KOSE	FDSC	Kao
n	27			136
Sensitivity (%)	88	94	94	88
Specificity (%)	**60**	**60**	**50**	66
Accuracy (%)	74	82	78	82

GHS	Phase	Chemical name	Lab.A	Lab.B	Lab.C
NC	I-A	Diethylphthalate	P	P	P
		Sodium lauryl sulfate	P	P	P
	I-B	Hexane	N	N	N
		Dextran	N	N	N
		Tween 80	N	N	N
	I-C	Lactic acid	N	N	N
	II	Acetanisole	P	N	P
		1-Iodohexane	P	P	P
		propylene glycol	N	P	P
		Benzylbutylphthlate	N	N	N
1A	I-A	Glyoxal	P	P	P
		Lauryl gallate	N	N	N
	I-B	2,4-Dinitrochlorobenzene	P	P	P
	I-C	Methyl heptine carbonate	P	P	P
		p-Phenylenediamine	P	P	P
	II	Tetrachlorosalicylanilide	P	P	P
		Isoeugenol	P	P	P
		2-Aminophenol	P	P	P
		Glutaraldehyde	P	P	P
1B	I-A	Benzisothiazolinone	N	P	P
	I-B	Ethyl acrylate	P	P	P
	I-C	Farnesol	P	P	P
		Abietic acid	P	P	P
	II	Lilial	P	P	P
		Methyl methacrylate	P	P	P
		Amyl cinnamic aldehyde	P	P	P
		Imidazolidinyl urea	P	P	P

Overall predictivity based on Kao's data

EpiSensA	Validation			Dataset
	LION	KOSE	FDSC	Kao
n	27			136
Sensitivity (%)	88	94	94	88
Specificity (%)	**60**	**60**	**50**	**66**
Accuracy (%)	74	82	78	82

Predictive capacity (other test methods)

h-CLAT	Validation				Dataset
	Kao	Shiseido	Bioassay	ECVAM	TG
n	24				142
Sensitivity (%)	75	88	75	88	93
Specificity (%)	**63**	75	75	**50**	**66**
Accuracy (%)	71	83	83	75	85

IL-8 luc assay	Validation			Dataset
	Lab.A	Lab.B	Lab.C	TG
n	34			118
Sensitivity (%)	87	83	83	96
Specificity (%)	100	90	90	**41**
Accuracy (%)	91	88	82	86

U-SENS	Validation				Dataset
	L'Oreal	Bioassay	CiToxLAB	WIL Res.	TG
n	38 (Ring trial+validation)				166
Sensitivity (%)	100	95	100	95	91
Specificity (%)	89	100	74	95	**65**
Accuracy (%)	95	97	87	95	86

It is likely that specificity of EpiSensA fell within other test methods.

4. Future plan

Hajime appreciates the all. Thanks to all, the experiment parts of validation study are successful. He mentioned the future plan.

1) The first draft validation report is developed by Kao be the end of this year. He will provide this draft in January, 2022.
2) He is planning the F2F meeting in February, 2022. He is optimistic of effect vaccine for COVID-19.
3) He will share the invitation this autumn.