厚生労働科学研究費補助金（化学リスク研究事業）

化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化

令和２年度分担研究報告書

免疫毒性データの集積、国際標準化へ向けてのvalidation試験の計画、国際会議の企画、進行

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研究要旨

*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay : MITA)に含まれるIL-1β Lucアッセイ及び新たに相場らによって開発されたIL-2 Lucアッセイの変法であるIL-2 Luc LTT アッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development : OECD）の試験法ガイドライン（Test Guideline : TG）として公定化するため、国際バリデーション研究を施行した。本年度、IL-1β Lucアッセイに関しては、相場らの作成したバリデーション報告書案をもとにVMTで議論した。新たにバリデーションを開始したIL-2 Luc LTT アッセイについては、施設内及び施設間再現性を検証するため、バリデーション研究（phase I）を実施した。その結果、いずれの施設も目標値である80%を達成でき、追加物質で施設間再現性を検証するためのPhase IIに移行できた。

研究協力者氏名・所属機関名及び所属機関における職名

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A．研究目的

in vitro 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）に含まれる IL-1β Luc アッセイ及び新たに相場らによって開発された IL-2 Luc アッセイの変法である IL-2 Luc LTT アッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドライン（Test Guideline：TG）として公定化するため、国際バリデーション研究を施行する。

B．研究方法

B-1. IL-1β Lucアッセイのバリデーション研究の報告書の作成

免疫毒性データを集積し、IL-1β Luc assay(国際バリデーション研究 phase I、IIが既に終了)の最終結果を反映した報告書案が相場らによって、MITAに関する国際バリデーション実行委員会（Validation Management Team: VMT、表１）に提出された。なお、この報告書の中で、私はデータの品質管理（Quality Control: QC）にあたる部分の記載とappendixの作成を担当した。

本報告書内容の合意を得るため、VMT電話会議を企画した。

B-2. IL-2 Luc LTT アッセイバリデーション研究の実験支援

B-2-1. IL-2 Luc LTT アッセイバリデーション被験物質の送付

VMTの電話会議でバリデーション計画について議論した後、IL-2 Luc LTT アッセイの技術移転性を評価するため、Phase0(以下、Phase 0と記す)として3物質を各施設に配布した。次に、Phase I (以下、Phase Iと記す)のために選ばれた5物質をコード化し、施設内及び施設間再現性を評価するために各施設に送付した。

また、Phase I終了後、Phase II (以下、Phase IIと記す)のために選ばれた20物質をコード化し、施設間再現性を評価するために各施設に送付した。

被験物質は、電話会議にて、相場らが取得した実験結果のある物質を用いて選択された。

B-2-2. IL-2 Luc LTT アッセイバリデーション結果の記録確認

Phase I で用いられた各施設の記録用紙及びデータを回収し、まとめた。

C. 結果

C-1. IL-1β Lucアッセイのバリデーション研究の報告書作成

相場らが作成したバリデーション報告書案について、VMTにて検討した。この報告書の中で、QC報告書及びAppendixを作成した（添付資料1~3)。QCの結果、いずれもGLP（Good Laboratory Procedure）施設ではないが、すべての結果はプロトコルに準じて求められ、実験に関わるすべての記録も適切になされていることを確認した。

電話会議は7月21日（添付資料4）、9月8日（添付資料5）、1月7日（添付資料6）に開催され、主に本アッセイの免疫毒性における科学的な位置づけ、予測性、予測性値を改善するための方法などについて議論された。

本アッセイの指標であるIL-1βは自然免疫に関与するTol受容体に関与し、妥当な指標である合意は取れた。しかし、in houseデータを用いて予測値を検討したところ、まだVMTの合意は得られておらず、議論が続いている。

C-2. バリデーション研究の実験支援

C-2-1. IL-2 Luc LTTアッセイバリデーション被験物質の送付

VMTにて新たな試験法であるIL-2 Luc LTTアッセイバリデーション計画を議論し、その計画（添付資料7）が合意された。まず技術移転性を評価するため、Phase0として3物質を各施設に配布した(表2)。Phase0で良好な技術移転性を確認した後（添付資料8）、Phase Iとして施設間再現性を求めるためにVMTにて選ばれた5物質を、コード化してリード施設を含む参加3施設に送付した。表3に被験物質のコード番号を示す。結果としてPhase Iにおける各施設の結果はすべて一致し、いずれの施設も施設内及び施設間再現性の目標値である80%を達成できた(結果は本報告書には示していない)。

なお、Phase IIが進行中であることもあり、各施設に配布した被験物質名は本報告書ではまだ公開できない。

実験の終了まで、被験物質による誤使用などによる健康障害などのトラブルは生じなかった。

C-2-2. IL-2 Luc LTTバリデーション結果の記録確認

Phase I終了後に回収した記録用紙の一覧を添付資料9に示した。施設によって一部記載の不備があったが、GLPの精神に則り、適切に実験が実施され、その記録が残されていることを確認し、VMTに提示した。

D. 考察

MITAの一つであるIL-1β Lucアッセイの予測性は、現状では高いものではない。大森らが統計学的な処理を行って検討したが、予測性を高めることができなかった。特に、特異度が低いことから、バリデーション報告書の完成にはもう少し時間が必要と考えている。

一方、MITAのもう一つの試験法であるIL-2 Luc LTTアッセイのバリデーション研究のphase Iは無事終了した。追加実験なく、phase IIに移行することができた。

来年度以降、Phase IIの終了を受け、バリデーション報告書が作成され、第三者評価委員会に移行されることを期待している。

いずれの方法も将来的には、OECDにてTGと採択されることを目指しており、来年度にはいずれの方法もOECDに提案できる段階となると考えている。

E. 結論

相場らにより開発されたMITAに含まれるIL-1β Lucアッセイ及びIL-2 Luc LTTアッセイの公定化を目指すため、国際的なバリデーション研究を施行した。IL-1β Lucアッセイについては、バリデーション報告書をVMTで議論している。新たにバリデーションを開始したIL-2 Lucアッセイの変法であるIL-2 Luc LTTアッセイに関しては、Phase Iにおいていずれの施設も施設内及び施設間再現性の目標値である80%を達成でき、追加物質で施設間再現性を検証するためのPhase IIに移行できた。

F. 添付文書

1) Quality assurance report for IL-1β validation study
2) Appendix 17-1 IL-1β(P1)2018 Check List
3) Appendix 17-2 IL-1β(P2)2019 Check List
4)Minutes, Conference call for the MITA assay validation study on July 21th, 2020
5)Minutes, Conference call for the MITA assay validation study on September 8th, 2020
6)Minutes, Conference call for the MITA assay validation study on January 7th, 2021
7) Study plan for the validation trial on multicolor reporter assay using IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) as a test evaluating the immunotoxic potential of chemicals
8) Results of Phase 0
9) IL-2 LTT (P1)2020 Check List

表１．2020年度　MITA国際バリデーション実行委員会及び参加施設の主なリスト

No.	Name	Affiliation	Country
1	Emanuela Corsini	Universit.AN` degli Studi di Milano	Italy
2	Erwin L. Roggen	3Rs Management and Consulting ApS	Denmark
3	Dori Germolec	NIH/NIEHS	USA
4	Tomoaki Inoue	Chugai Pharmaceutical Co., Ltd.	Japan
5	Setsuya Aiba	Tohoku University Graduate School of Medicine	Japan
6	Yutaka Kimura	Tohoku University Graduate School of Medicine	Japan
7	Yoshihiro Nakajima	National Institute of Advanced Industrial Science and Technology (AIST), Shikoku	Japan
8	Rie Yasuno	AIST, Tsukuba	Japan
9	Takashi Omori	Kobe University	Japan
10	Nana Mashimo	Kobe University	Japan
11	K. Okayama	Kobe University	Japan
12	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan

表2　IL-2 LTTアッセイ　Phase 0 被験物質リスト

	Chemical name	CAS No.	Storage	Physicality	Supplier
1	Bleomycin sulfate	9041-93-4	C(0～10°C)	Solid	TCI
2	6-Thioguanine	154-42-7	R	Solid	TCI
3	Dexamathasone	50-02-2	C(2～10°C)	Solid	WAKO

表3　IL-2 LTTアッセイ　Phase I 被験物質コード表

IL-2 LTT_P1 Coded Chemicals

No.	LabA Tohoku			LabB Tsukuba			LabC Shikoku		
	set1	set2	set3	set1	set2	set3	set1	set2	set3
1	MLA102	MLA204	MLA303	MLB402	MLB504	MLB602	MLC705	MLC802	MLC903
2	MLA101	MLA202	MLA304	MLB404	MLB503	MLB604	MLC701	MLC804	MLC905
3	MLA104	MLA205	MLA305	MLB401	MLB505	MLB603	MLC702	MLC805	MLC902
4	MLA105	MLA203	MLA301	MLB405	MLB502	MLB601	MLC704	MLC803	MLC904
5	MLA103	MLA201	MLA302	MLB403	MLB501	MLB605	MLC703	MLC801	MLC901

G. 研究発表

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H. 知的所有権の取得状況

H－1）特許取得

特になし

H－2）実用新案登録

特になし

H－3）その他

特になし

Quality assurance report for IL-1β validation study

Hajime Kojima and Asako Ueda

JaCVAM, NIHS

2020.3.31

１．Chemical distribution

1-1. Chemical Acquisition, Coding and Distribution

The assessment of laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were performed with the coded chemicals. The coding was supervised by JaCVAM (See Appendix 1). JaCVAM was responsible for coding and distributing the test chemicals for the validation study.

1-2. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing the hazards identification and exposure controls/personal protection for each chemical (See Appendix 2.1 and 2.2). The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

２． Quality assurance

All the records (data sheets and record sheets) from the participating laboratories were checked by Dr. Takashi Omori, Kobe univ. and JaCVAM (See Appendix 3). The record sheets mean “Reagent records, solubility test, Cell culture records, Test records and data sheets”. They are total more than 300 pages and available at JaCVAM website (http:// http://www.jacvam.jp/validation08-login.html). Testings performed as part of a validation study were carried out in accordance with the principles of GLP (OECD, 1998) and necessarily include, without being limited to, the use of protocol and adequate recording of data as well as suitable reporting of results and archival record keeping.

The culture of the cells, the preparation and application of test chemicals and data sheets were completed and the results accurately reflect the raw data. Unfortunately, the record sheets on the maintenance of measuring instruments had not collected before the

validation study. JaCVAM considered these records had concerns on quality of data in the validation study. However, JaCVAM checked carefully all the results and judged all data within acceptable ranges.

At least, the reliability of measuring instruments would be checked by an independent organization before the validation study. JaCVAM recommend the validation management team the formal validation study participated with GLP laboratories will be done.

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IL-1β (P1)2018 Check List

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set1	Reagent Records	IL-1 2018-A01	IL-1 2018-B01	IL-1 2018-C01
	Solubility Test	IL-1 2018-A02	IL-1 2018-B02	IL-1 2018-C02
	Cell Culture Records	IL-1 2018-A03	IL-1 2018-B03	IL-1 2018-C03
	Date	2018.12.7	2019.1.23	2019.1.22
	Test Records	IL-1 2018-A04	IL-1 2018-B04	IL-1 2018-C04
	Datasheets	IL-1 2018-A41	IL-1 2018-B41	IL-1 2018-C41
	Date	2018.12.10	2019.1.29	2019.1.24
	Test Records	IL-1 2018-A05	IL-1 2018-B05	IL-1 2018-C05
	Datasheets	IL-1 2018-A42	IL-1 2018-B42	IL-1 2018-C42
	Date	2018.12.14		2019.1.28
	Test Records	IL-1 2018-A06		IL-1 2018-C06
	Datasheets	IL-1 2018-A43		IL-1 2018-C43
	Date	2018.12.17		
	Test Records	IL-1 2018-A07		
	Datasheets	IL-1 2018-A44		
	Date	2018.12.21		
	Test Records	IL-1 2018-A08		
	Datasheets	IL-1 2018-A45		
	Date	2018.12.26		
	Test Records	IL-1 2018-A09		
	Datasheets	IL-1 2018-A46		
	Date	2018.12.28		
	Test Records	IL-1 2018-A10		
	Datasheets	IL-1 2018-A47		
	Date	2019.1.11		
	Test Records	IL-1 2018-A11		
	Datasheets	IL-1 2018-A48		
	Date	2019.1.28		
	Test Records	IL-1 2018-A12		
	Datasheets	IL-1 2018-A49		
set1 retrial	Date		2019.5.13	
	Test Records		IL-1 2018-B10	
	Datasheets		IL-1 2018-B43	

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set2	Reagent Records	IL-1 2018-A21	IL-1 2018-B21	IL-1 2018-C21
	Solubility Test	IL-1 2018-A22	IL-1 2018-B22	IL-1 2018-C22
	Cell Culture Records	IL-1 2018-A23	IL-1 2018-B03	IL-1 2018-C23
	Date	2019.2.18	2019.1.30	2019.1.31
	Test Records	IL-1 2018-A24	IL-1 2018-B24	IL-1 2018-C24
	Datasheets	IL-1 2018-A51	IL-1 2018-B51	IL-1 2018-C51
	Date	2019.2.20	2019.2.8	2019.2.4
	Test Records	IL-1 2018-A25	IL-1 2018-B25	IL-1 2018-C25
	Datasheets	IL-1 2018-A52	IL-1 2018-B52	IL-1 2018-C52
	Date	2019.2.25	2019.2.12	2019.2.12
	Test Records	IL-1 2018-A26	IL-1 2018-B26	IL-1 2018-C26
	Datasheets	IL-1 2018-A53	IL-1 2018-B53	IL-1 2018-C53
	Date	2019.2.27		
	Test Records	IL-1 2018-A27		
	Datasheets	IL-1 2018-A54		
	Date	2019.2.28		
	Test Records	IL-1 2018-A28		
	Datasheets	IL-1 2018-A55		
	Date	2019.3.1		
	Test Records	IL-1 2018-A29		
	Datasheets	IL-1 2018-A56		
	Date	2019.3.4		
	Test Records	IL-1 2018-A30		
	Datasheets	IL-1 2018-A57		
set2 retrial	Cell Culture Records	IL-1 2018-A73		IL-1 2018-C73
	Date	2019.5.9		2019.5.27
	Test Records	IL-1 2018-A70		IL-1 2018-C70
	Datasheets	IL-1 2018-A71		IL-1 2018-C71

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set3	Reagent Records	IL-1 2018-A31	IL-1 2018-B31	IL-1 2018-C31
	Solubility Test	IL-1 2018-A32	IL-1 2018-B32	IL-1 2018-C32
	Cell Culture Records	IL-1 2018-A33	IL-1 2018-B03	IL-1 2018-C33
	Date	2019.3.11	2019.2.14	2019.2.18
	Test Records	IL-1 2018-A34	IL-1 2018-B34	IL-1 2018-C34
	Datasheets	IL-1 2018-A61	IL-1 2018-B61	IL-1 2018-C61
	Date	2019.3.13	2019.2.18	2019.2.21
	Test Records	IL-1 2018-A35	IL-1 2018-B35	IL-1 2018-C35
	Datasheets	IL-1 2018-A62	IL-1 2018-B62	IL-1 2018-C62
	Date	2019.3.14	2019.3.3	2019.2.25
	Test Records	IL-1 2018-A36	IL-1 2018-B36	IL-1 2018-C36
	Datasheets	IL-1 2018-A63	IL-1 2018-B63	IL-1 2018-C63
	Date	2019.3.15		
	Test Records	IL-1 2018-A37		
	Datasheets	IL-1 2018-A64		
	Date	2019.3.18		
	Test Records	IL-1 2018-A38		
	Datasheets	IL-1 2018-A65		
set3 retrial	Cell Culture Records		IL-1 2018-B03	IL-1 2018-C73
	Date		2019.2.28	2019.5.31
	Test Records		IL-1 2018-B70	IL-1 2018-C80
	Datasheets		IL-1 2018-B71	-
	Date			2019.6.3
	Test Records			IL-1 2018-C90
	Datasheets			IL-1 2018-C91

IL-1β (P2)2019 Check List

	LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
Reagent Records	IL-1 2019-A01	IL-1 2019-B01	IL-1 2019-C01
Solubility Test	IL-1 2019-A02	IL-1 2019-B02	IL-1 2019-C02
Cell Culture Records	IL-1 2019-A03	IL-1 2019-B03	IL-1 2019-C03
Date	2019.9.2	2019.8.19	2019.8.16
Test Records	IL-1 2019-A04	IL-1 2019-B04	IL-1 2019-C04
Datasheets	IL-1 2019-A20	IL-1 2019-B20	IL-1 2019-C20
Date	2019.9.4	2019.8.20	2019.8.19
Test Records	IL-1 2019-A05	IL-1 2019-B05	IL-1 2019-C05
Datasheets	IL-1 2019-A21	IL-1 2019-B21	IL-1 2019-C21
Date	2019.9.6	2019.8.22	2019.8.22
Test Records	IL-1 2019-A06	IL-1 2019-B06	IL-1 2019-C06
Datasheets	IL-1 2019-A22	IL-1 2019-B22	IL-1 2019-C22
Date	2019.9.9	2019.8.23	2019.8.23
Test Records	IL-1 2019-A07	IL-1 2019-B07	IL-1 2019-C07
Datasheets	IL-1 2019-A23	IL-1 2019-B23	IL-1 2019-C23
Date	2019.9.12	2019.8.26	2019.8.26
Test Records	IL-1 2019-A08	IL-1 2019-B08	IL-1 2019-C08
Datasheets	IL-1 2019-A24	IL-1 2019-B24	IL-1 2019-C24
Date	2019.9.13	2019.8.27	2019.8.29
Test Records	IL-1 2019-A09	IL-1 2019-B09	IL-1 2019-C09
Datasheets	IL-1 2019-A25	IL-1 2019-B25	IL-1 2019-C25
Date	2019.10.3	2019.8.29	2019.9.2
Test Records	IL-1 2019-A10	IL-1 2019-B10	IL-1 2019-C10
Datasheets	IL-1 2019-A26	IL-1 2019-B26	IL-1 2019-C26
Date	2019.10.4	2019.9.2	2019.9.5
Test Records	IL-1 2019-A11	IL-1 2019-B11	IL-1 2019-C11
Datasheets	IL-1 2019-A27	IL-1 2019-B27	IL-1 2019-C27
Date		2019.9.6	2019.9.6
Test Records		IL-1 2019-B12	IL-1 2019-C12
Datasheets		IL-1 2019-B28	IL-1 2019-C28
Date		2019.9.9	2019.9.9
Test Records		IL-1 2019-B13	IL-1 2019-C13
Datasheets		IL-1 2019-B29	IL-1 2019-C29
Date			2019.9.12
Test Records			IL-1 2019-C14
Datasheets			IL-1 2019-C30

Draft minutes

Conference call for the MITA assay validation study

Date: July 21th, 2020

Validation Management Team (VMT): Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Nakajima, Y., Yasuno, R., Kojima, H.

1. Welcome address and approve draft agenda
   Kojima welcomed to join this meeting and the VMT members approved the agenda.
2. Predictivity of IL-1β assay
   Based on discussion the predictive capacity on IL-1β assay in the last meeting, Aiba proposed a new hypothesis on the correlation with TLR signaling and IL-1β. TLR signaling activates NF-κB and MAP kinase to induce regulatory responses in any stimulated receptor. The active NF-κB promotes the transcription of NF-κB-dependent genes, such as NLRP3, Pro-IL-1 and Pro-IL-18, which are necessary for inflammasome activation. On the other hand, the transcription factor NF-κB regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses such as IL-2 and IL-1. NF-κB plays a critical role in regulating the survival, activation and differentiation of inflammatory T cells. So, it makes a sense for the reason that there are similar results between IL-2 and IL1β regarding NF-κB. The VMT agreed with this hypothesis.

   In addition, Aiba explained it is important to investigate different signaling pathways for immunotoxicity and the IL-1β assay supports out of applicability domain in the IL-8 assay. So, we should have same options based on different mechanisms in IATA such as endpoints of IL-2, IL-1 and IL-8. The VMT agreed with his proposal which is included in the detailed review paper.

   Kojima proposed Aiba will be shared the all members the tables and results in the validation report by the next meeting in September.
3. Proposal of new protocol and study plan for IL-2 Luc assay LTT
   Aiba introduced the protocol of IL-2 Luc assay LTT. Especially, he talked about the prediction model and proposed five categories; Leukocyte toxic, Augmentation, immunosuppression, equivocal and no effects. The VMT concerned these complicated criteria for the validation study. They requested more simple criteria and proposes the definition of positive means the inhibition of cell proliferation and the others are defined as negative. Aiba agreed this prediction model for the validation study.

   Kojima introduced the draft revised study plan on the validation study for IL-2 Luc assay LTT and invited the all members at the VMT. He proposed to avoid the tight

schedule. The validation study will be finished phase I by the end of this year and completed phase II one year later. He suggests to start a pre-validation study using three chemicals; bleomycin, 6-thioguanine and dexamethasone next month.

After the pre-validation study, he organised the next meeting in September and we discussed the revised protocol based on the results and the only VMT will select the chemicals for phase I. The members agreed this proposal.

Draft minutes

Conference call for the MITA assay validation study

Date ： September 8th, 2020

Validation Management Team: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Kojima, H.

Chemical selection for phase I

After leaving the delegates of the participated laboratories, the VMT members discussed the chemical selection. Firstly, Kojima proposed the criteria of selecting candidate chemicals for phase I. In addition, Kojima proposed nine candidate chemicals for phase I.

- Five chemicals are selected for phase I.
- Mainly, the candidates should be selected from the list of Detailed Review Paper for in vitro immunotoxicity.
- The candidates should be selected from the chemicals tested by the test developers.
- The candidates should be selected at least each one in four classifications.

1) IL-2 Luc: positive, IL-2 Luc LTT: positive
2) IL-2 Luc: positive, IL-2 Luc LTT: negative
3) IL-2 Luc: negative, IL-2 Luc LTT: positive
4) IL-2 Luc: negative, IL-2 Luc LTT: negative

All the members discussed the selection criteria refer to the chemical list. As a result, all members made an agreement as the following; 1) The candidates were selected at least each one in four classifications.2)Regardless metabolism products, it is important for metabolic activity, 3)Regardless in vivo MOA, in vitro results based on in house data are put emphasis, 4) For within-laboratory reproducibility, three positives and two negatives are selected, and 5) Two liquids and three solids are selected based on the information physical properties.
Finally, all the members agreed the following draft candidate chemicals for phase I. On the other hand, Emanuela suggested more additional chemical or replacement one to Indeterminate (water insoluble chemical) in prediction model. Kojima will share the draft list and in house data to all after the meeting and continue to discuss the final decision by e-mail. He wished to distribute the chemicals by the end of September and start phase 1 next month. So, he hopes to fix the chemicals for phase I ASAP.

1． Mycophenolic acid, CAS. 24280-93-1, Solid positive, Immunosuppressive drugs, myelo or leukocyte-toxic

2. Indomethacin, CAS. 53-86-1, Solid, positive, Immunosuppressive drugs
3. Cyclosporin A, CAS. 59865-13-3, Solid, negative, Immunosuppressive drugs
4. 5-FU, CAS. 51-21-8, Solid, positive, Anti-caner drug/Immunosuppressive drugs, myelo or leukocyte-toxic
5. Ethanol, CAS. 64-17-5, Liquid, negative, Immunosuppressive drugs*

*I am sorry that Mycophenolic acid is a solid compound. The balance of liquid: solid is too bad in phase I. This is my proposal and I would like to change from ethanol to cyclophosphamide. In this case, there are all solid chemicals used in phase I.

Draft minutes

Conference call for the MITA assay validation study

Date ： January 7th, 2021

Validation Management Team: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Y., Omori, T., Nakajima, Y., Yasuno, R., Kojima, H.

1. Welcome address and approve draft agenda
   Dr. Kojima welcomed to all and draft agenda was approved.

2. IL-2 Luc LTT
   Omori introduced the results of phase I. The obtained data from three laboratory was perfect.
   Kimura proposed a minor change to phase II. The VMT approved the phase I results and minor modification of protocol.
   Kojima mentioned the experiment parts of phase I completed and to move phase II in February. After the members left this meeting, he proposed the VMT the candidate list of phase II. Hopefully, he requested them to fix the chemicals for phase II by the middle of January.

3. IL-1 Luc assay validation report.
   Aiba introduced about the validation report. Corsini pointed out of error predictivity combined with IL-2 Luc assay and IL-1 Luc assay. He requested the comments and suggestions to all, because he has not yet found the good predictive capacity. After this meeting, the following comments shared.

please find attached the document with some comments. As it is written it may generate confusion in the External Reviewers. To me WLR, BLR and predictive capacity must presented separately: Phase I WLR; Phase II BLR; and predictive capacity including all results. It must be clearly stated that the purpose of the validation study was to assess transferability, WLR and BLR. I see that having changed the prediction model after Phase I, we wanted to evaluated again WLR also in Phase II but this needs to be clearly stated. In any case, I will leave predictive capacity as a separate chapter.

For the overall predictive capacity, one should refer to the Lead lab. It is also important to mention that the assay will not be used a stand alone assay but as a tool in a toolbox. In addition, considering that since a specific parameter is measured it has been difficult to find in vivo data that specifically measured IL-1, which resulted in an apparent low predictive capacity. Then, I like the approach used to establish it, with the history of the process.

My apologies for taking longer to get to this than I had agreed. Attached please find my comments on the report. I agree with Emanuela's comments below. In addition, I found that often, even though a call of Stimulatory or Augmentation was made, it was not referred to in the writeup. I think that the document needs to be more clear as to when augmentation was measured, how it was calculated and there needs to be some additional discussion outside of the summary box of why the criteria were changed. Hajime and I were just on a review panel where there was much discussion about the transparency of using test chemicals and data from multiple phases in the final analyses, and so I have tried to note where perhaps this should be clarified. Please let me know if you have any questions, I am also happy to discuss if needed.

I'm sending my general comments on the issues when mRNA expressions are applies as only endpoints of toxicities without measuring protein functions. It may depend on the target proteins and cell types.

In the case of IL-1b, the IL-1b is transcripted as a precursor of IL-1b which is inactive form of IL-1b. The precursor is processed by caspase-1 to form active form of IL-1b. Effects of chemicals on caspase-1 may affect the IL-1b activities. The activity of proteasomes including caspase-1 may be different depending on cell types. In the IL-1b Luc assay, THP-1 cells are used, but IL-1b is secreted also from other cell types such as neutrophils, B-cells, endothelial cells, NK cells. The activation system of IL-1b may be different depending on the cell types.

I actually experienced in my research on human iPS-derives hepatocytes that CYP mRNA expressions are not indications of CYP activities.

遅くなりました．いくつか検討しましたが，なかなかうまくはいかないですね．
〇以前提示されていた IL1-beta と Il2 の OR の組み合わせ
と今回の検討 1
感度：77.6%　特異度：14.3%
〇今回の検討 2
感度：87.8%　特異度：0%
と芳しくありません．ちなみに他の組み合わせもやりましたが，上記を超えるような成績にはなりませんでした．
データを見ていて，気が付いたのは，
・組み合わせの予測で”P”となってしまうものが多いこと
・Immunotoxicity judgment が”Negative”の物質はわずか 14 物質ということです．
このデータでは，あまりにも 2 つの系で P になっているのですが，もっと広い範囲で免疫毒性とは無縁の物質に適用したときに，どちらかが p になるようなことがあるのでしょうか?そうなると試験系としては問題ということになるのですが，素人の私としてはそんなに光るわけがないように思います．

”Positive”物質が 49 あるので，もし免疫毒性がない一般の物質で，IL1-beta も IL2 も N になるような物質をあと 35（＝49-14）行われたら，特異度は上がるはずで，OR 判定なら 14.3%は，75.5%（＝100×（2＋35）/49）までは上がると思います

Study plan
for the validation trial on multicolor reporter assay using IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) as a test evaluating the immunotoxic potential of chemicals

Version 1.0 July, 2020

Conducted by:

IL-2 Luc assay Validation Management Team

INDEX

1. Background

The use of multicolor reporter assay using IL-2 Luc, Jurkat cell (IL-2 assay) is an important for evaluating the immunotoxic potential of chemicals as a part of Multi-ImmunoTox assay (MITA), because of its technical simplicity, short-term test period and accuracy of test result based on a mechanism of immunotoxicity.

The aim of this trial is to (pre)validate the modified IL-2 Luc assay, IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) method to assess transferability and inter-laboratory variability, in order to incorporate this test for screening the immunotoxic chemicals. This assay has developed to support the evidence of IL-2 Luc assay. The IL-2 Luc LTT for the validation trial will be undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004], iii) according to the concept discussed on the validation trials with participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of the validation trials is described in the context of GLP, iv) and in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies part of a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, but not necessary limited, use of standard operating procedures (SOP), adequate data recording, reporting and record keeping are essential.

A general conceptional framework [Hartung et al., 2004; OECD, 2005] will be used for documenting all the study to assess the validation status of a test method, called "modular approach" to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition

Module 2: Within-laboratory repeatability and reproducibility

Module 3: Between-laboratory transferability

Module 4: Between-laboratory reproducibility

Module 5: Predictive capacity

Module 6: Applicability domain

Module 7: Performance standards

The Modular approach as introduced by Hartung et al., allows using datasets from various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc LTT. This IL-2 Luc LTT for the validation

trial has performed under the GLP principle.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc LTT with a challenging set of test substances (test items) for which high quality *in vitro and in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc LTT Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
~~Shihori Tanabe~~	~~Chemical supplier~~	~~JaCVAM, NIHS, Japan (JaCVAM representative)~~
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
JSIT liaison	Immunotoxicity expertise	Chugai Pharmaceutical Co.,

Tomoaki Inoue		Ltd.

3.1 Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Tohoku University. Study Director (SD): Yutaka Kimura

Test Facility 2: AIST, Tsukuba SD: Rie Yasuno

Test Facility 3: AIST, Takamatsu SD: Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc LTT is transferable and reproducible between experienced laboratories. The all facility will be the laboratory participating in this validation trial acting as unexperienced laboratory to assess between laboratory transferability, reliability and relevance of the IL-2 Luc LTT method under non-GLP conditions (GLP principle).

3.2 Trial management structure

1) Chemical management group

The members of chemical management group are elected by recommendation of the IL-2 Luc LTT VMT. They prepare a tentative list of test chemicals and works with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed are distributed by JaCVAM.

2) Data analysis group

The members of data analysis group are elected by recommendation of the IL-2 Luc LTT VMT, and check and analyze the data obtained in this validation trial from a third-party standpoint. They also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of record management group are elected by recommendation of the IL-2 Luc LTT VMT. They prepare protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the eventually necessary data recording or calculation

templates. The Trial Coordinator has to ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

3.3 Sponsor

The validation trial for assessing the validity of IL-2 Luc LTT will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc LTT validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- the financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- the test, reference and control item purchase, coding and distribution to the test facility
- the availability of the test systems to the participating laboratories by supporting the Lead laboratory with the logistics for delivering the test system to the facility
- the independent data analysis and statistical support (biostatistician) based on the study reports generated
- the other costs for participating laboratories

3.4 Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc LTT validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator’s responsibilities include:

a) Establishment of/support to lead laboratory, including meeting organization
b) Trial communication and coordination with test facilities
c) Recording of document and data flow between test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, Study Plans and Study Reports
h) The communication of the results of the trial into the public domain

The role of Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial coordinator (unless otherwise communicated by the participating Test Facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the trial coordinator and the Lead Laboratories) and, if appropriate, validated data recording, data analysis, data reporting sheets for the test method.

It is the responsibility of the Trial Coordinator to approve the study plans send for approval by the test facilities, and any amendments to the study plan, by dated signature.

3.5 Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to the all test facility giving details what training aspects will be covered during the training of the other SDs and Study Personnel at the lead laboratory. Furthermore, after the training as Phase 0 study, the lead laboratory will issue to the Trial Coordinator a training report and indicating if critical observations are made by the other test facilities regarding the IL-2 Luc LTT protocols. In case any critical observations are made a new version of the IL-2 Luc LTT protocols might

necessary be issued to the other test facilities before initiating the between-laboratory transferability.

3.6 [Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of the all test facility has been done by an independent biostatistical analysis using coded five chemicals, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I study.

3.7 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

For the transfer of IL-2 Luc LTT to the all test facility, the Phase 0 study using - coded five chemicals were performed. A few concentrations of each test item will be tested in triplicate in 3 independent runs according to the IL-2 Luc LTT protocol describing the details of the experimental design.

The five test items selected for the phase I study are coded as follows: A, B, C, D, and E. The all facility will prepare a study according to internal GLP principle. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability will be reviewed before progressing with module 4 on the between laboratory reproducibility. If the transferability data do not meet test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the Trial Coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle).

3.8 [Module 4] Between-laboratory reproducibility

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibility in the phase I and II study. A few concentrations of each test item will be tested in triplicate according to the IL-2 Luc LTT protocol describing the details of the experimental design.

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and

data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria,

3.9 [Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data of the between laboratory reproducibility has been assessed. Depending on the statistical analysis the lean design for validation as well as the automatisation of the test leading to an increased dataset will be considered.

4. Protocol

In this validation trial, the protocol (ver. 0.01E, phase I) will be used). This protocol will make up a draft by the lead laboratory and be finalized by VMT. The criteria to identify immunotoxicants by MITA are provisionally fixed in the protocol ver. 0.01E prior to the phase I study. There are two temporary criteria to identify imunotoxicans. The VMT adopted the criteria after the phase I validation study.

A measurement of bioluminescence intensity induced with chemical treatment will be measured by luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

5. Chemicals

5.1 Chemical Selection

Test chemicals have been selected by chemical repository based on published papers on in vivo immunotoxicity

The applied selection criteria were:

- information on mode/site of action
- coverage of range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high quality data derived from animals and (if available) also humans
- knowledge on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of range of toxic effects/potencies
- chemicals that do not need metabolic activation
- appropriate negative and positive controls

- physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- costs

In the first phase of the selection procedure, the Chemical management group identified and collected several existing lists of potential chemical sensitizing in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes e.g. as reference compounds for validation studies. An extensive literature research was performed by the Chemical management group insuring that the preselected chemical fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase IL-2 Luc LTT validation trial with data generation at the test facilities, five chemicals will be tested three times in each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of Phase I results, detailed test planning of the Phase II will be determined. At this moment, twenty chemicals will be planned in the phase II trial for predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase 0	3 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

5.2 Chemical Acquisition, Coding and Distribution

The assessment of within-laboratory reproducibility (Module 2), between laboratory transferability (Module 3) in the all test facilities have been performed with coded chemicals. This IL-2 Luc LTT validation trial plan describes the generation of the missing data sets under coded test item. If the results obtained are not very similar to

the previous obtained sets, the VMT has to assess if coded chemicals need to be tested in the all test facilities.

The coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible of coding and distribution of test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive the safety information concerning the hazards identification and exposure controls/personal protection.

6. Records and archiving

At the end of the trial, the IL-2 Luc LTT validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

7. Study timeline

An approximate schedule for IL-2 Luc LTT validation trial is shown in Table 3.

Duration of this validation trial is around twenty -month from July 2020 to August

2021.

Table 3. Schedule of IL-2 Luc LTT validation trial

Month	Activity
July, 2020	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
August, 2020	Technical transfer using three known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
September, 2020	Coding and distribution of five coded test chemicals
September, 2020	Start of Phase I study
December, 2020	End of Phase I study
February, 2021	**1st VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
April, 2021	Coding and distribution of coded test chemicals and positive chemicals
May, 2021	Start of Phase II study using 20 coded test chemicals
August,2021	End of Phase II study
November-December, 2021	**2nd VMT Meeting** /reviewing of Phase II study results
Q2, 2022	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

6-Thioguanine

1st Exp.

2nd Exp.

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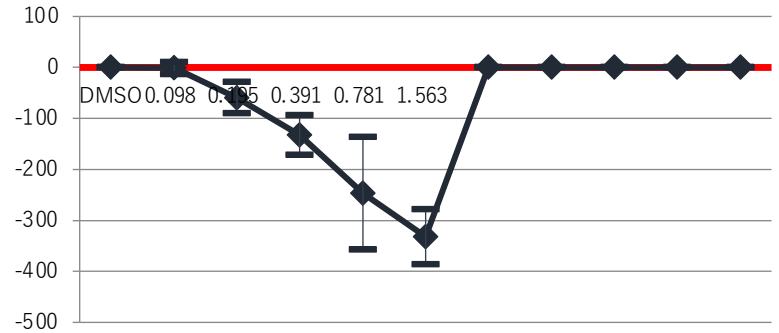
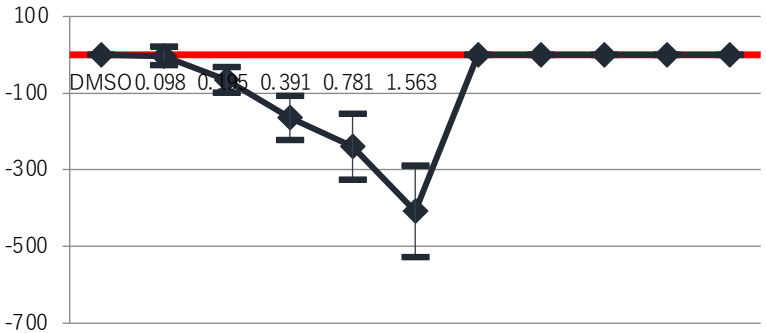
TOHOKU





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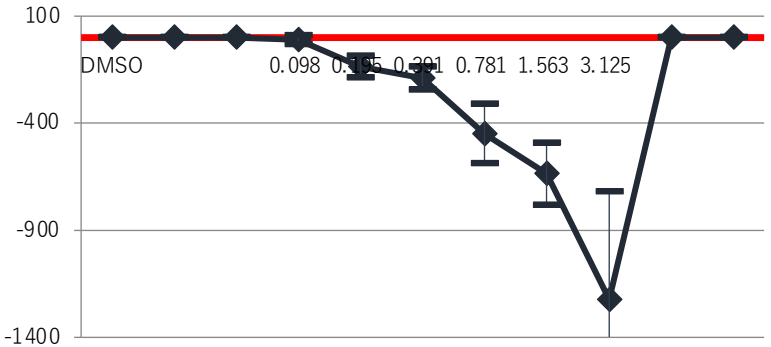

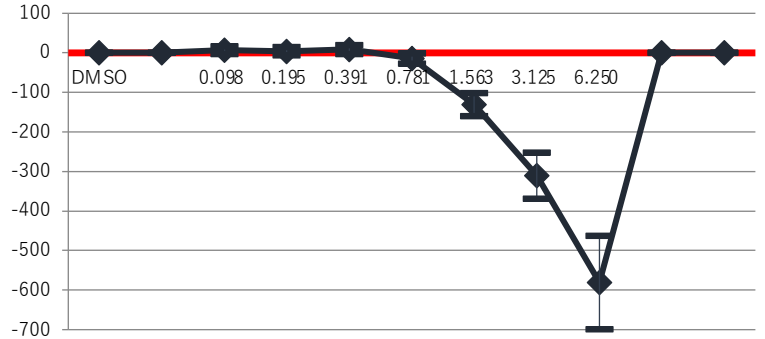

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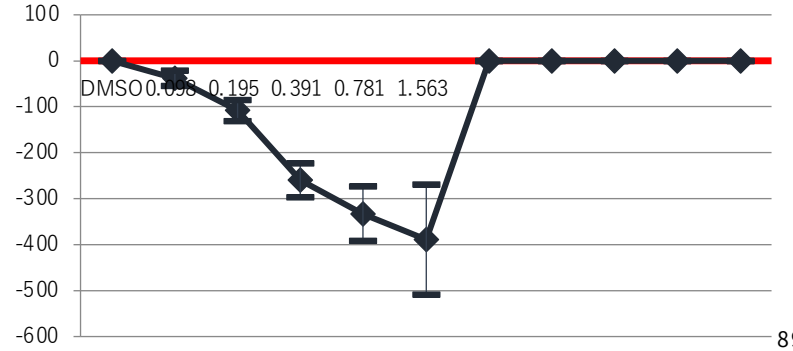

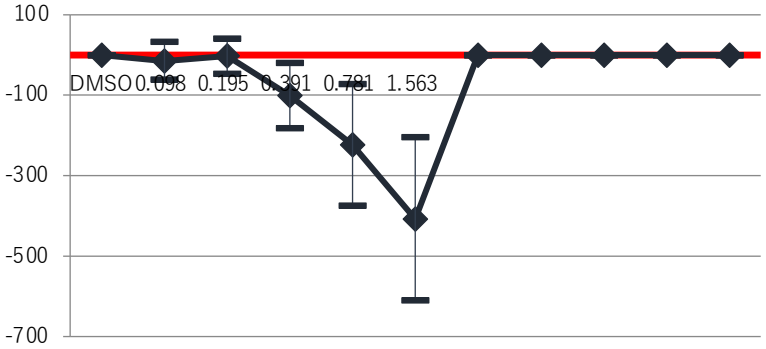

Bleomycin sulfate

IL-2 LTT P0

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TSUKUBA

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1st Exp.

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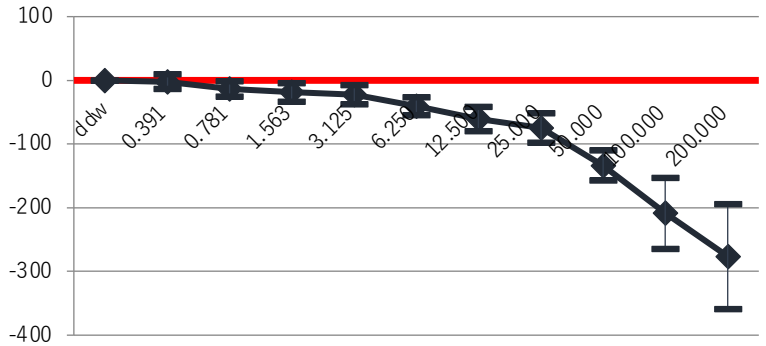

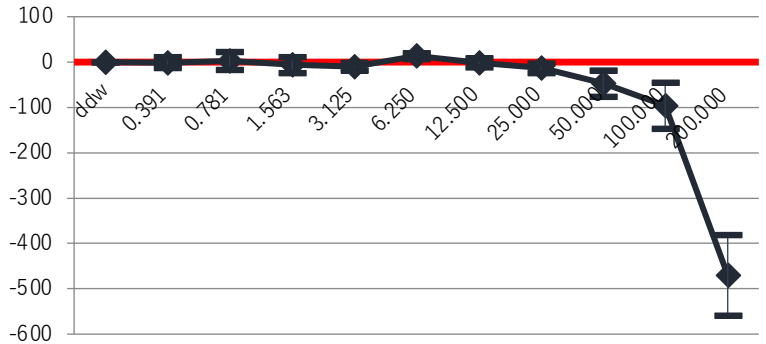

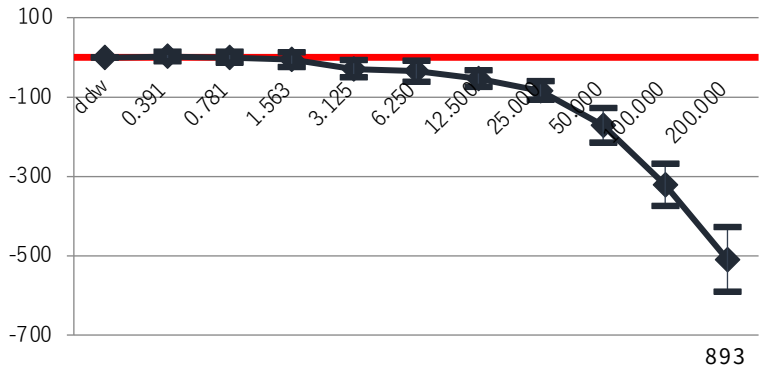

2nd Exp.

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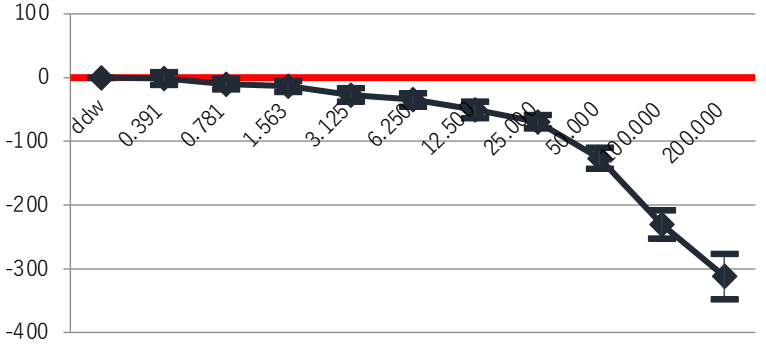

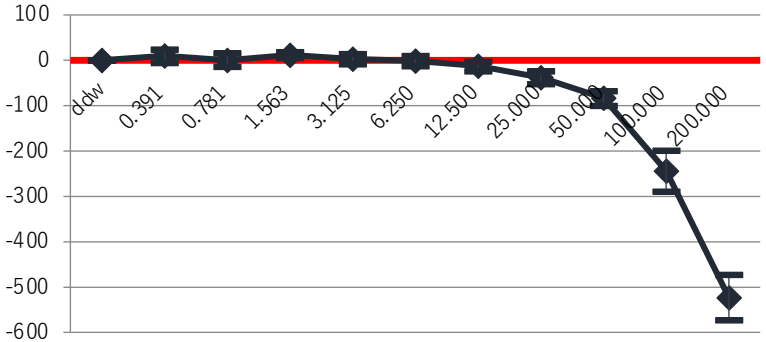

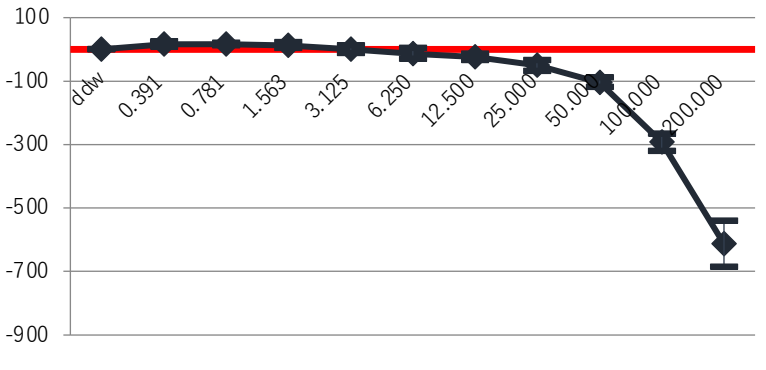

Dexamethasone

IL-2 LTT P0

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SHIKOKU

1st Exp.

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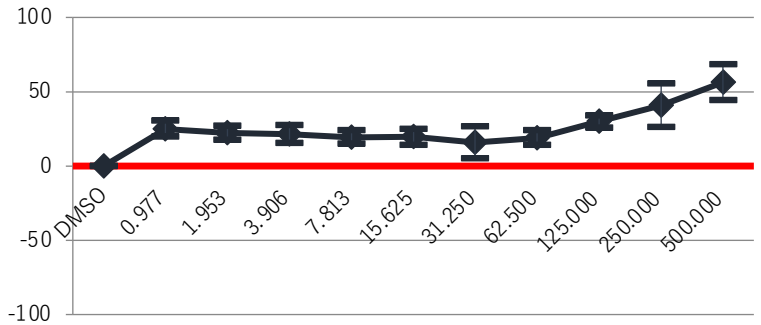

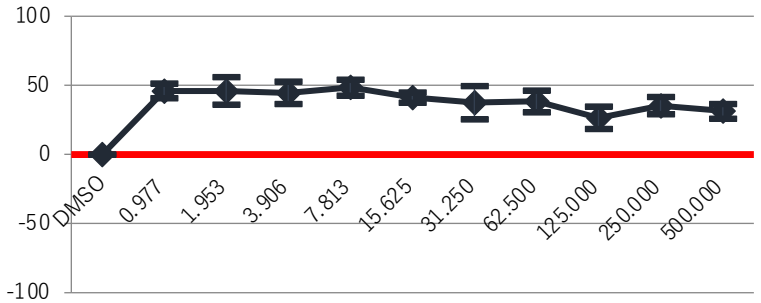

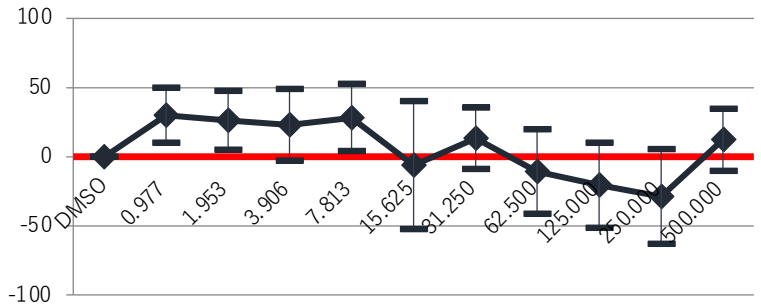

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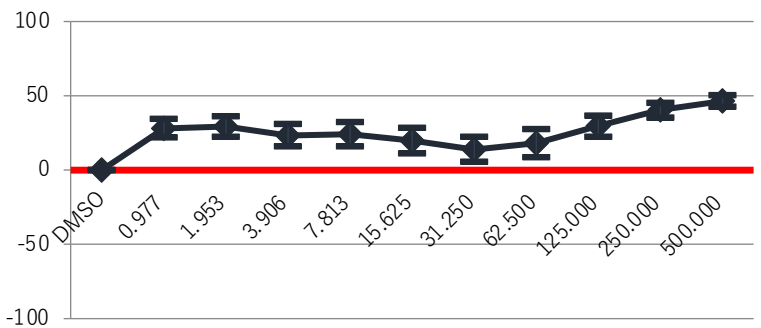
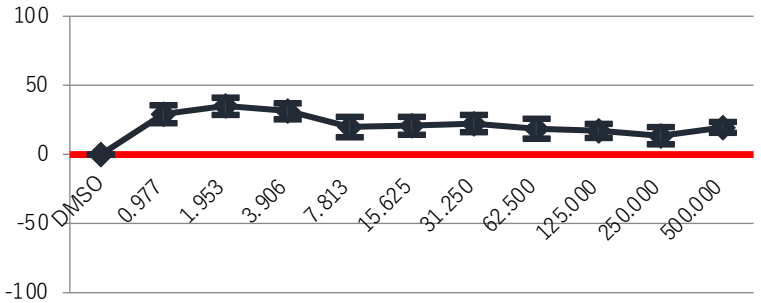
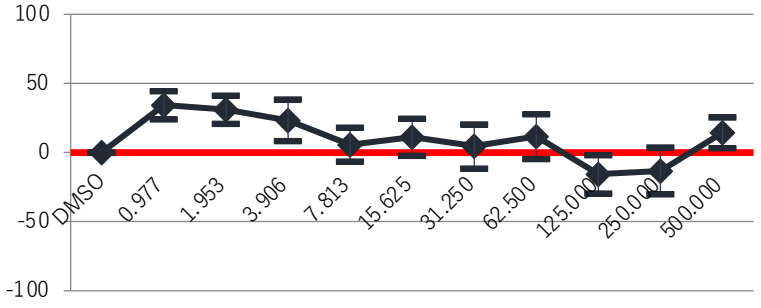






IL-2 LTT(P1)2020 Check List

2021.2.24

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set1	Reagent Records	IL-2 2020 A01	IL-2 2020 B01	IL-2 2020 C01
	Solubility Test	IL-2 2020 A02	IL-2 2020 B02	IL-2 2020 C02
	Cell Culture Records	IL-2 2020 A03	IL-2 2020 B03	IL-2 2020 C03
	Date	2020.10.26	2020.10.29	2020.10.22
	Test Records	IL-2 2020 A04	IL-2 2020 B04	IL-2 2020 C04
	Datasheets	IL-2 2020 A31	IL-2 2020 B31	IL-2 2020 C31
	Date	2020.10.29	2020.11.2	2020.10.26
	Test Records	IL-2 2020 A05	IL-2 2020 B05	IL-2 2020 C05
	Datasheets	IL-2 2020 A31	IL-2 2020 B31	IL-2 2020 C31
set2	Reagent Records	IL-2 2020 A11	IL-2 2020 B11	IL-2 2020 C11
	Solubility Test	IL-2 2020 A12	IL-2 2020 B12	IL-2 2020 C12
	Cell Culture Records	IL-2 2020 A03	IL-2 2020 B03	IL-2 2020 C03
	Date	2020.11.4	2020.11.4	2020.10.29
	Test Records	IL-2 2020 A14	IL-2 2020 B14	IL-2 2020 C14
	Datasheets	IL-2 2020 A41	IL-2 2020 B41	IL-2 2020 C41
	Date	2020.11.9	2020.11.9	2020.11.5
	Test Records	IL-2 2020 A15	IL-2 2020 B15	IL-2 2020 C15
	Datasheets	IL-2 2020 A41	IL-2 2020 B41	IL-2 2020 C41
set3	Reagent Records	IL-2 2020 A21	IL-2 2020 B21	IL-2 2020 C21
	Solubility Test	IL-2 2020 A22	IL-2 2020 B22	IL-2 2020 C22
	Cell Culture Records	IL-2 2020 A03	IL-2 2020 B03	IL-2 2020 C03
	Date	2020.11.12	2020.11.5	2020.11.12
	Test Records	IL-2 2020 A24	IL-2 2020 B24	IL-2 2020 C24
	Datasheets	IL-2 2020 A51	IL-2 2020 B51	IL-2 2020 C51
	Date	2020.11.16	2020.11.11	2020.11.16
	Test Records	IL-2 2020 A25	IL-2 2020 B25	IL-2 2020 C25
	Datasheets	IL-2 2020 A51	IL-2 2020 B51	IL-2 2020 C51
Bleo	Reagent Records	IL-2 2020 A60	IL-2 2020 B60	IL-2 2020 C60
	Datasheets	IL-2 2020 A61	IL-2 2020 B61	IL-2 2020 C61
Dex	Reagent Records	IL-2 2020 A60	IL-2 2020 B60	IL-2 2020 C60
	Datasheets	IL-2 2020 A62	IL-2 2020 B62	IL-2 2020 C62

国立医薬品食品衛生研究所
小島　肇　様

試験番号: GP-JKE-172005
報告書作成日:2021年3月29日

試験報告書

ご依頼の試験を実施し、下記の結果を得ましたのでご報告いたします。

【試験標題】　HACベクター導入IL-8 THP細胞のFISH解析

【試験期間】　2021年2月25日～2021年3月29日

株式会社ジーピーシー研究所
〒683-0826　鳥取県米子市西町86番地
とっとりバイオフロンティア内
TEL 0859-21-7232

責任者	担当者	担当者

試験報告書

【試験標題】

HACベクター導入IL-8 THP細胞のFISH解析

【試験内容】

HACベクター上に搭載したGAPDHSLR:IL8SLO遺伝子を保持するTHP-1細胞の核型解析（FISH）を実施する。

【試験期間】

2020年7月29日～2021年2月26日

【試験概要】

デュアルレポータシステム用プラスミドを搭載したHACベクターを保持するTHP-1IDAC細胞について、核型標本を作製し、Fluorescence in situ hybridization （FISH） 解析を行う。

【使用機器・試薬等】

細胞培養関連

- THP-1IDAC細胞
- RPMI medium, Gibco, cat#11875093
- FBS, Sigma, cat#172012-500ML, lot#14L369
- Penicillin/Streptomycin, 富士フィルム和光純薬, cat#168-23191
- G418（50 mg/mL）, ナカライテスク, cat#084-07681
- Blasticidin S （10 mg/mL） , cat#A1113903
- Nick translation Mix, Roche, cat#111745808910
- Biotin-16-UTP,Roche, cat#1138908910
- Avidin-FITC, Roche, cat＃
- その他関連試薬

試薬・機器関連

染色体解析顕微鏡, カールツァイスマイクロスコピー, Imager Z2
恒温槽, TAITEC, THERMO MINDER SJ-07N

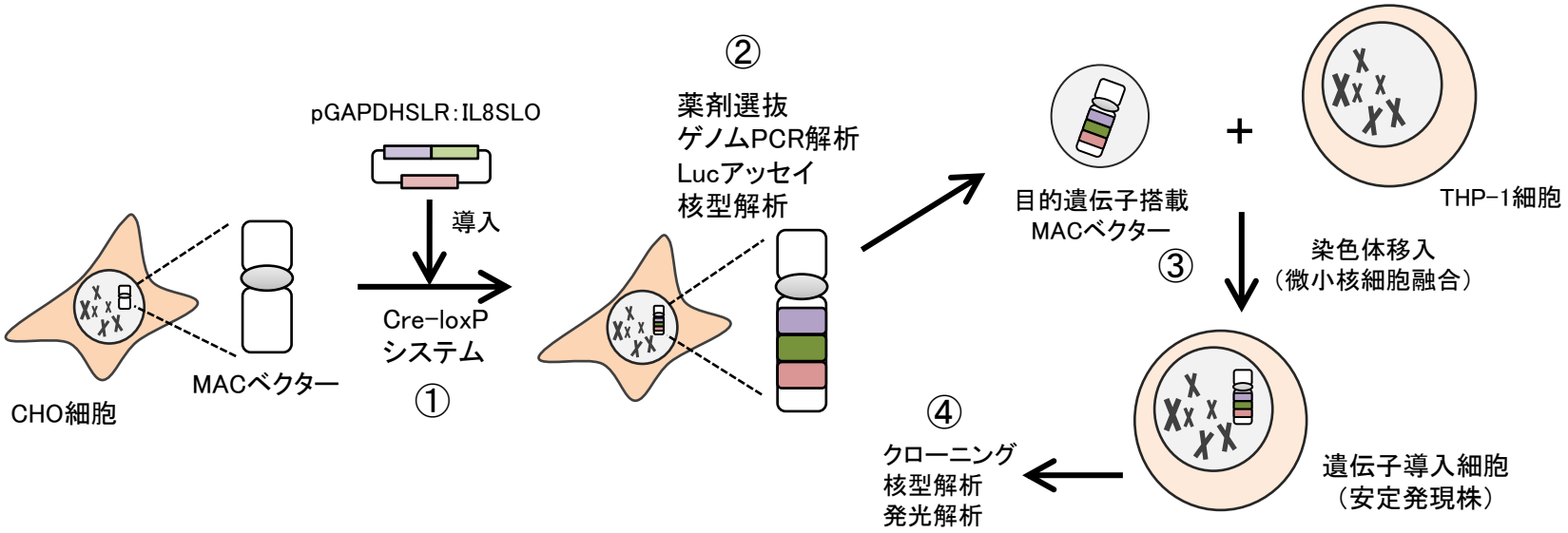
【試験方法】

1. HACベクター上に搭載されたGAPDHSLR:IL8SLO(GAPDHR-IL8O) 遺伝子を保持するTHP細胞の核型解析

1-1 HACベクター保持THP細胞をちいて核型標本を作製する。
1-2 GAPDHR-IL8O遺伝子をプローブとして、Fluorescence in situ hybridization(FISH)により、HACベクター上への遺伝子搭載の有無を確認する。

【報告書一覧】

【GAPDHSLR:IL8SLO(GAPDHR-IL8O) 遺伝子を保持するTHP細胞作製　概要】



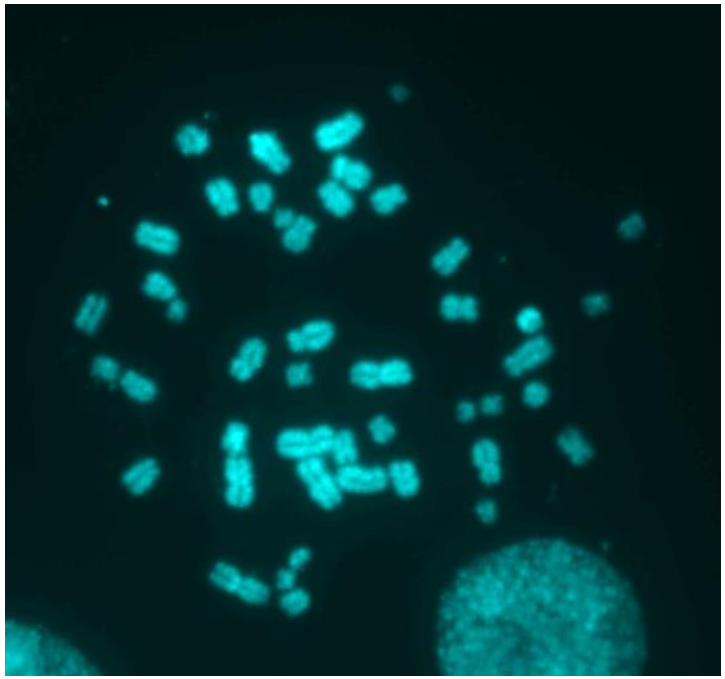
① Cre-loXPシステムを用いたHACベクターへのプラスミド(pGAPDHSLR:IL8SLO)の搭載。
② 薬剤選抜による細胞単離を実施。ゲノムPCRおよびLucアッセイによる薬剤応答性評価による候補クローンの選抜。
③ 微小核融合法(MMCT)によるTHP-1 IDAC細胞へのHACベクター移入。
④ クローニングした細胞における遺伝子保持確認(PCR・核型解析)および薬剤応答性解析。

試験結果

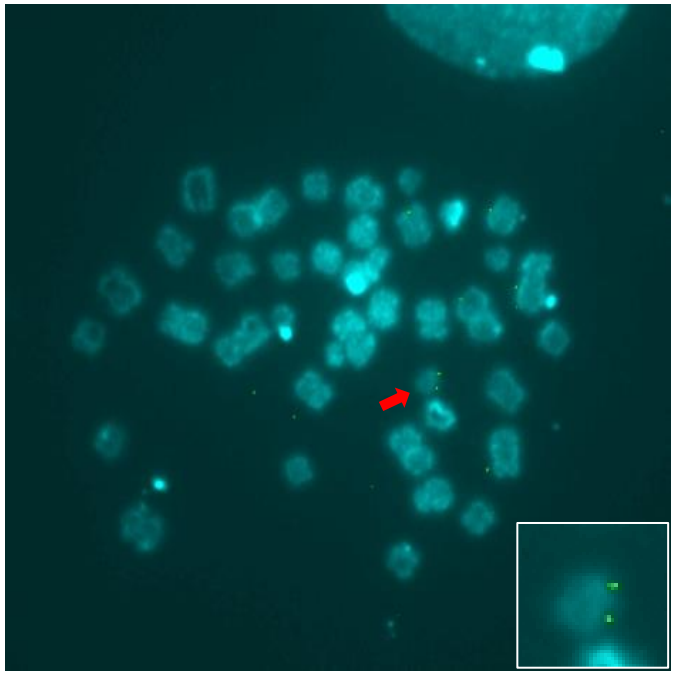
【 1. HACベクター上に搭載されたGAPDHSLR:IL8SLO（GAPDHR-IL8O） 遺伝子を保持するTHP細胞の核型解析】

・ GAPDHR-IL8O 遺伝子導入THP-1 IDAC細胞バルクより単離したクローン細胞＃2（PCRにてGAPDHR-IL8O遺伝子導入を確認済み）について、GAPDHR-IL8Oプラスミドをプローブとして、FISH解析を実施した。

FISH結果



THP-1 IDAC (WT)



クローン　2

赤矢印：HACベクター
白枠：HACベクター拡大
プローブ：
GAPDHSLR:IL8SLO
プラスミド

THP-1 IDAC細胞内において、GAPDHR-IL8O遺伝子搭載HACベクターが1個保持されていることを確認できた。

総括

HACベクター上にGAPDHSLR:IL8SLO遺伝子を搭載したTHP-1 IDAC細胞バルクより単離したクローンの内、搭載遺伝子およびプロモーター領域のPCRを実施し、目的の導入遺伝子およびプロモーター領域が導入されていることを確認できたものについて、核型標本を作製し、FISH解析を行った。

その結果、THP-1 IDAC細胞内で、GAPDHSLR:IL8SLO遺伝子を搭載したHACベクターが1個保持されていることが確認できた。一方、本細胞を用いて実施したLucアッセイでは、発光を検出することができていないという結果も得られており、今後は、バルク細胞群からシングルクローニング作業を継続し、それらについてLucアッセイ評価を実施し、薬剤応答性の高いクローンを単離する必要がある。

国立医薬品食品衛生研究所
小島　肇　様

試験番号: GP-JKE-202005
報告書作成日:2021年2月26日

試験報告書

ご依頼の試験を実施し、下記の結果を得ましたのでご報告いたします。

【試験標題】　デュアルレポーターシステム搭載HACベクター導入THP-1細胞の構築

【試験期間】　2020年7月29日～2021年2月 25日

株式会社ジーピーシー研究所
〒683-0826　鳥取県米子市西町86番地
とっとりバイオフロンティア内
TEL 0859-21-7232

責任者	担当者	担当者

902

試験報告書

【試験標題】

デュアルレポーターシステム搭載HACベクター導入THP-1細胞の構築

【試験内容】

GAPDHSLR:IL8SLO遺伝子をHACベクターに搭載し、
これを保持するTHP-1細胞を作製する。

【使用機器・試薬等】

細胞培養関連

・CHO-H8細胞
・THP-1IDAC細胞
・Ham's F12, 富士フィルム和光純薬, cat#087-08335
・RPMI medium, Gibco, cat#11875093
・FBS, Sigma, cat#172012-500ML, lot#14L369
・Penicillin/Streptomycin, 富士フィルム和光純薬, cat#168-23191
・G418(50 mg/mL), ナカライテスク, cat#084-07681
・Blasticidin S (10 mg/mL), cat#A1113903
・Cytochalasin B, 富士フィルム和光純薬, cat#034-17554
・Cell Banker, TAKARA, cat#CB011
・その他汎用実験試薬、消耗品

【試験期間】

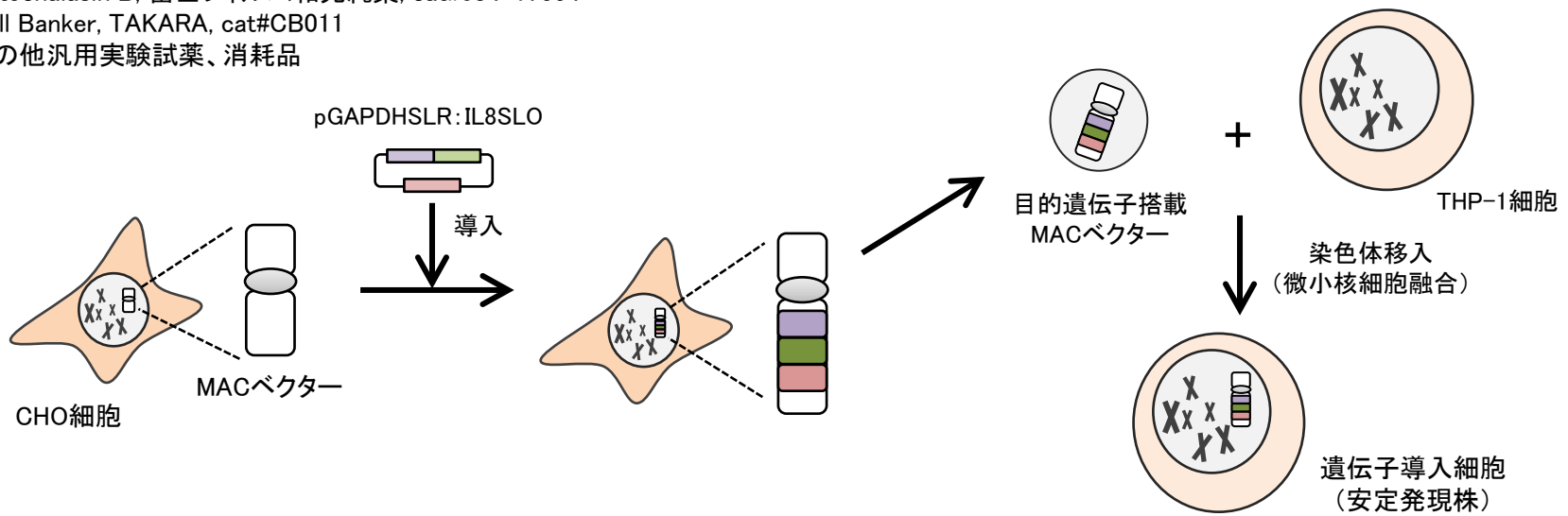
2020年7月29日～2021年2月26日

【試験概要】

デュアルレポータシステム用に構築されたプラスミドをCHO細胞内に保持されたHACベクターに搭載したのち、微小核細胞融合法を用いてTHP-1IDAC細胞に移入する。

試薬・機器関連

・KOD FX Neo, TOYOBO, cat#KFX-201
・Cell Lysis Solution, QIAGEN, cat#158906
・Protein Precipitation Solution, QIAGEN, cat#158910
・FHERIOS, ATTO
・Kronos H, ATTO
・Thermal Cycler Dice Touch, TaKaRa Bio



試験報告書

【試験方法】

1. HACベクターへのGAPDHSLR:IL8SLO（GAPDHR-IL8O） 遺伝子搭載

1-1 HACベクター保持CHO細胞を培養する。
1-2 プラスミド（CMV-LoxP:GAPDHR:IL8O:をHACベクターに搭載する。
1-3 G418で薬剤選抜を行い、耐性クローンを獲得する。
1-4 耐性クローンは、PCRにより染色体上へのGAPDHR-IL8O遺伝子移入を確認する。

2. GAPDHR-IL8O遺伝子搭載HACベクター保持細胞の機能評価

2-1 取得したGAPDHR-IL8O保持細胞候補クローンについて、TNFα及びIL-1応答性評価および核型解析を実施する。
2-2 TNFαまたはIL1 を添加した培地で2日間の培養を行い、発光量の変動をクロノスHT（ATTO）を用いて測定する。
2-3 各クローンの核型標本を作製し、HACベクターを保持していることを確認する。
2-4 TNF-αおよびIL-1応答性が良く、HACベクターが独立して保持されていることが確認されるクローンを選択する。

3. GAPDR-IL8O遺子搭伝載HACベクタ-のTHP-1細胞への移入

3-1 選択した細胞が十分に増えたタイミングでコルセミド添加培地に置換し、微小核を形成させる。
3-2 微小核を形成した細胞をサイトカラシン含有培地で満たし、遠心（8000rpm, 60min, 34℃）して、微小核を回収する。
3-3 ポリエチレングリコール（PEG）を用いて、THP1-IDAC細胞と微小核を融合し、HACベクターを移入する。
3-4 Blasticidin S で薬剤選抜を行い、耐性クローンを獲得する。
3-5 耐性クローンは、PCRにより染色体移入を確認する。

【報告書一覧】

試験報告書

【1:HACベクターへのGAPDHR-IL8O遺伝子搭載】

- Cre-loXPシステムを用いてHACベクターへのプラスミド(pGAPDHSLR:IL8SLO)の搭載を実施した。
- Ripofectamin 2000を用いてCMV-CreとpGAPDHSLR:IL8SLOを共導入した。
- G418 (500ug/mL)で薬剤選抜を実施し、60個のクローンを得た.。その内、先行して増殖したクローンについてゲノム抽出とPCRおよびLucアッセイを実施し、発光量が高く、遺伝子の導入が確認できた5クローン(R4, R6, W3, W7, W9)を選択した。

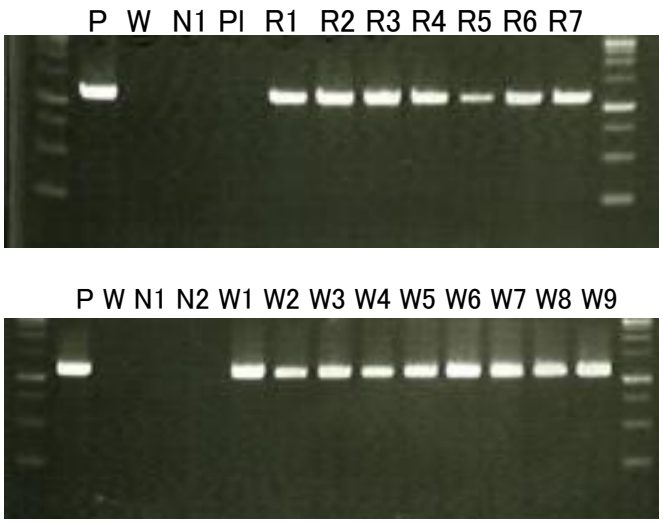
Luc アッセイ
20mM D-luciferin添加
全光(F0)　3秒測定

クローン名	発光値
R1	49
R2	30
R3	575
R4	2571
R5	65
R6	1284
R7	35
R8	252
R9	130
R10	240
R11	125
R13	124
R14	238
R15	1467
R16	36
R17	45
R18	154
R19	49
R20	40
R21	164
R22	138
R23	2575
R24	738

クローン名	発光値
w1	58
w2	44
w3	1947
w4	36
w5	63
w6	99
w7	1626
w8	68
w9	3087
w10	909
w11	1979
w12	3129
w13	1636
w14	640
w15	214
w16	17
w17	27
w18	367
w19	719
W20	53
W21	397
W22	2575
W23	21478

PCR結果

増幅領域 : HACベクター-プラスミド組換え部分(318bp)



・KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 1min
35 cycles

P: ポジティブコントロール
他遺伝子搭載HAC保持CHO細胞
W: H_2O
N1: ネガティブコントロール
HAC保持CHO細胞(WT)
Pl: 導入プラスミド
pGAPDHSLR:IL8SLO

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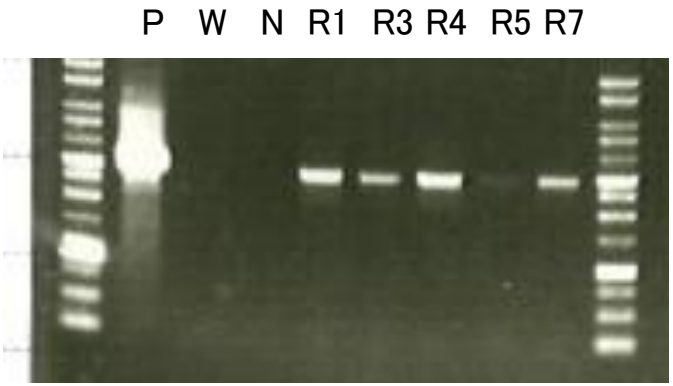
試験報告書

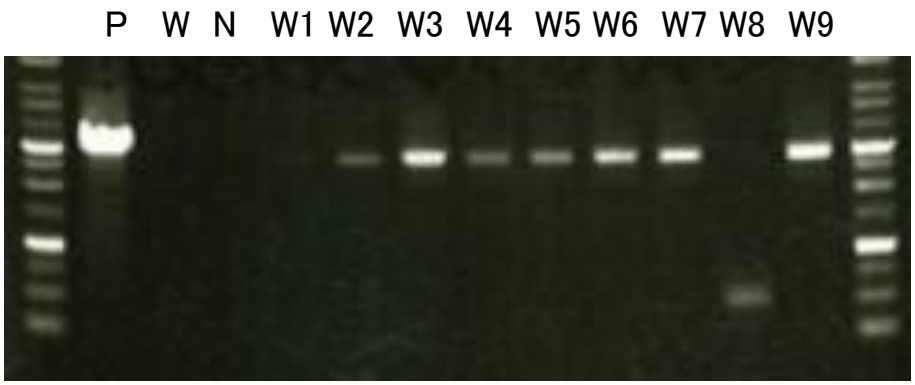
PCR結果

PI: 導入プラスミドpGAPDHSLR:IL8SLO
W: H_2O
N: ネガティブコントロール
HAC保持CHO(WT)

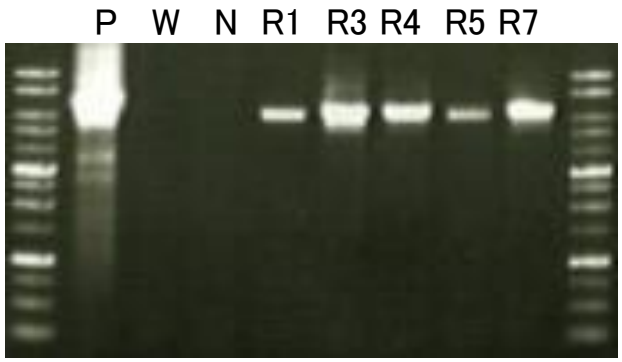
・KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 2min
35 cycles

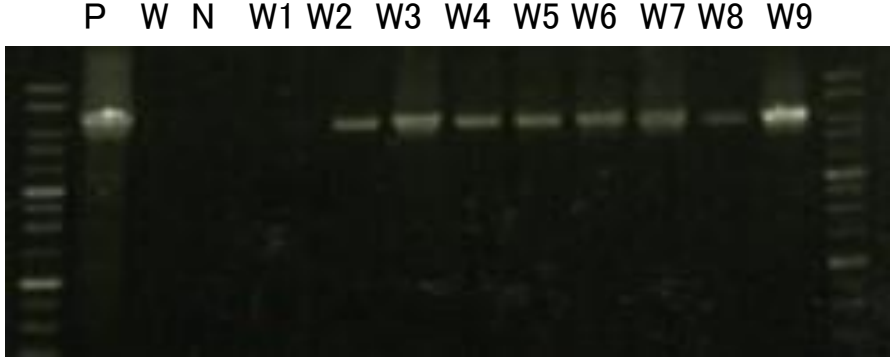
増幅領域: GAPDHプロモーター-SLR部分(3.0kb)





増幅領域: IL-8プロモーター-SLO部分(6.2kb)





W8を除く、クローンにおいてバンドが確認できた。

試験報告書

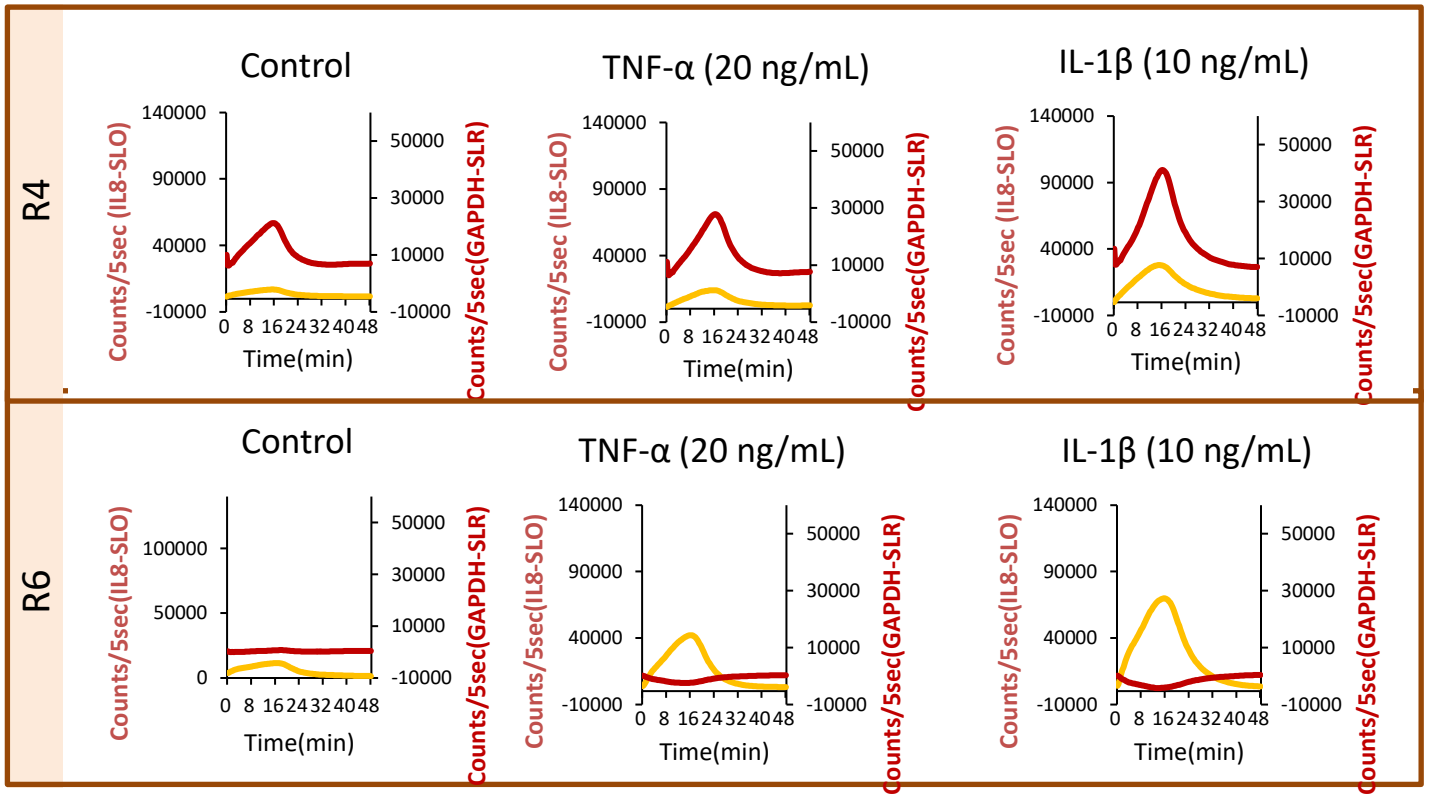
【2：GAPDHR-IL8O遺伝子搭載HACベクター保持細胞の機能評価TNFα・IL1応答性評価試験】

・PCRにて目的遺伝子の導入が確認でき、事前に実施したLucアッセイで高い蛍光値を示したHACベクター導入CHO細胞クローンについて下記条件で96well プレートに播種し、培地にTNFαおよびIL-1添加した際の発光量の変化を測定した。薬剤反応性と蛍光値および核型解析結果を考慮し、THP-1への微小核細胞誘導試験に用いるクローン（W3）を選択した。

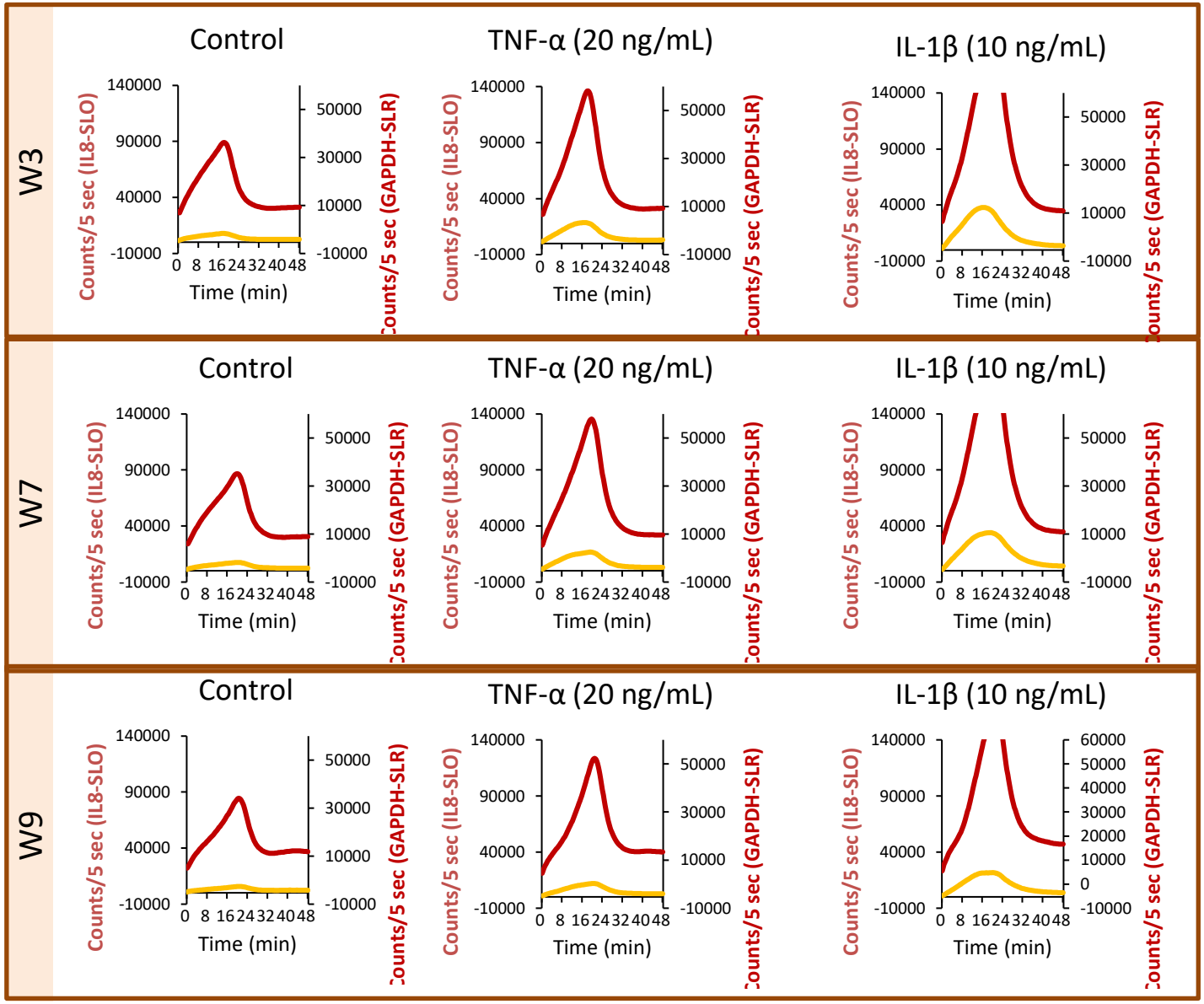
TNF-α・IL-1β応答性評価

試験条件

・ 5X103cells/wellで播種し、48時間後に各薬剤（TNF-α： 20 ng/mL, IL-1β：:: 100 μM10ng/mL, D-luciferin）を添加した培地に交換した。
・Kronos HTにてSLRおよびSLO発光量を48時間測定した(F0,F2, integral time 5sec, interval time min) 。



試験報告書



SLR・SLOの値が高W3, W7,W9を候補クローンとした。

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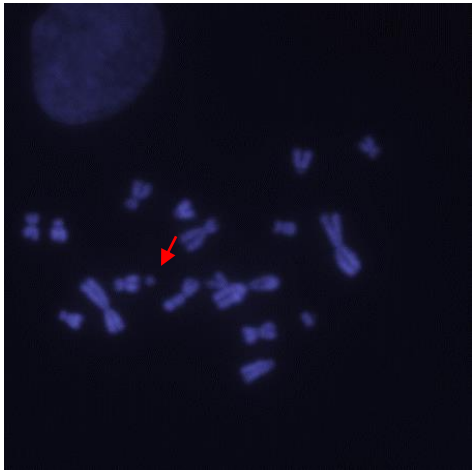
試験報告書

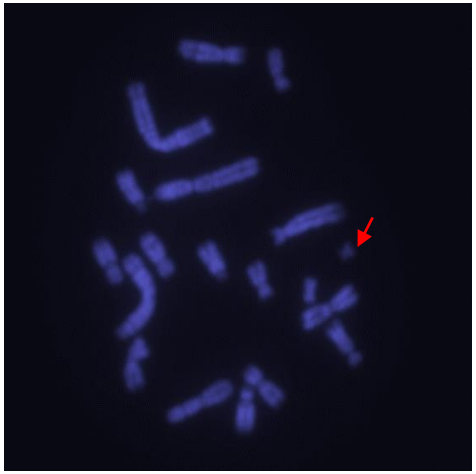
核型解析

・HACベクター保持CHO細胞 5クローン（R4, R6, W3, W7, W9)について、核型標本（DAPI染色）を作製し、宿主染色体本数及びHACベクター保持率を調べた。
・遺伝子が搭載されたHACベクターを確実にTHP細胞に移入するため、HACベクターが1個保持されているものとしてW3およびW9を候補クローンとして選択した。

W3



W9



	染色体本数（本）	HACベクター数（個）	metaphase数（個）	metaphase 20個中の割合
R4	13	1	1	5%
	17	2	1	5%
	18	1	2	10%
	23	1	1	5%
	30	1	3	15%
	30	2	4	20%
	32	1	1	5%
	32	3	1	5%
	34	1	1	5%
	34	2	6	30%
R6	17	2	8	40%
	18	1	10	50%
	19	1	2	10%
W3	18	1	20	100%
W7	18	1	15	75%
	19	1	3	15%
	35	0	1	5%
	37	1	1	5%
W 9	18	1	19	95%
	4 n	1	1	5%

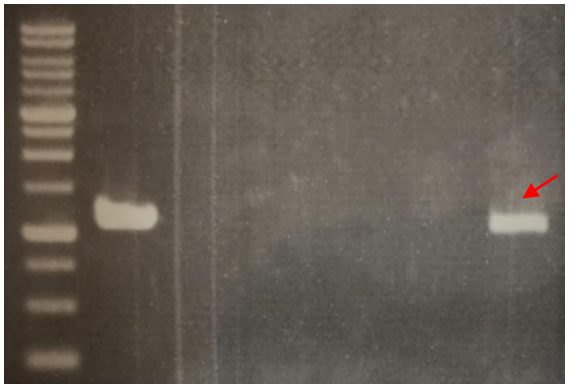
試験報告書

【3: GAPDHR-IL8O遺子搭伝載HACベクタ-のTHP-1細胞への移入移入】

・HACベクター保持CHO細胞 W3からの微小核細胞融合試験を実施した。
・ PEGを用いた染色体移入（細胞融合）後、細胞集団をBlasticidin S (6ug/mL)にて培養。細胞増殖後、ゲノム（バルク）を回収し、PCRを実施したところ、HACベクターの存在を確認できた。

サンプル（バルクゲノム）

P W N A B C



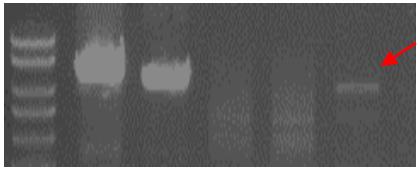
KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 1min
35 cycles

P: ポジティブコントロール
HAC保持CHO細胞 W3
W: H_2O
N: ネガティブコントロール
THP-1細胞（WT）
PI: 導入プラスミド
（pGAPDHSLR:IL8SLO）

増幅領域 : HACベクター-プラスミド組換え部分(318bp)

サンプル（バルクゲノム）

PI P W N C



KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 1min
35 cycles

増幅領域: IL-8プロモーター-SLO(6.2kb)

サンプル（バルクゲノム）

PI P N C

増幅領域: GAPDHプロモーター-SLR(3.0kb)

総括

CHO細胞内においてHACベクターにGAPDHSLR:IL8SLO遺伝子を搭載することができた。さらにこのHACベクターを微小核細胞融合法によりTHP-1 IDAC細胞に移入させることに成功した。

今後は、クローニング作業を実施し、導入遺伝子の動作確認（TNFα・IL1応答性評価試験）、THP-1IDAC細胞内におけるHACの保持状態確認（核型解析・FISH解析）及び遺伝子保持確認（PCR解析）へと順次検証を進めていくこととなる。