

**Bhas 42 Cell (*v-Ha-ras*-transfected BALB/c 3T3 clone)
transformation Assay to Predict Carcinogens**

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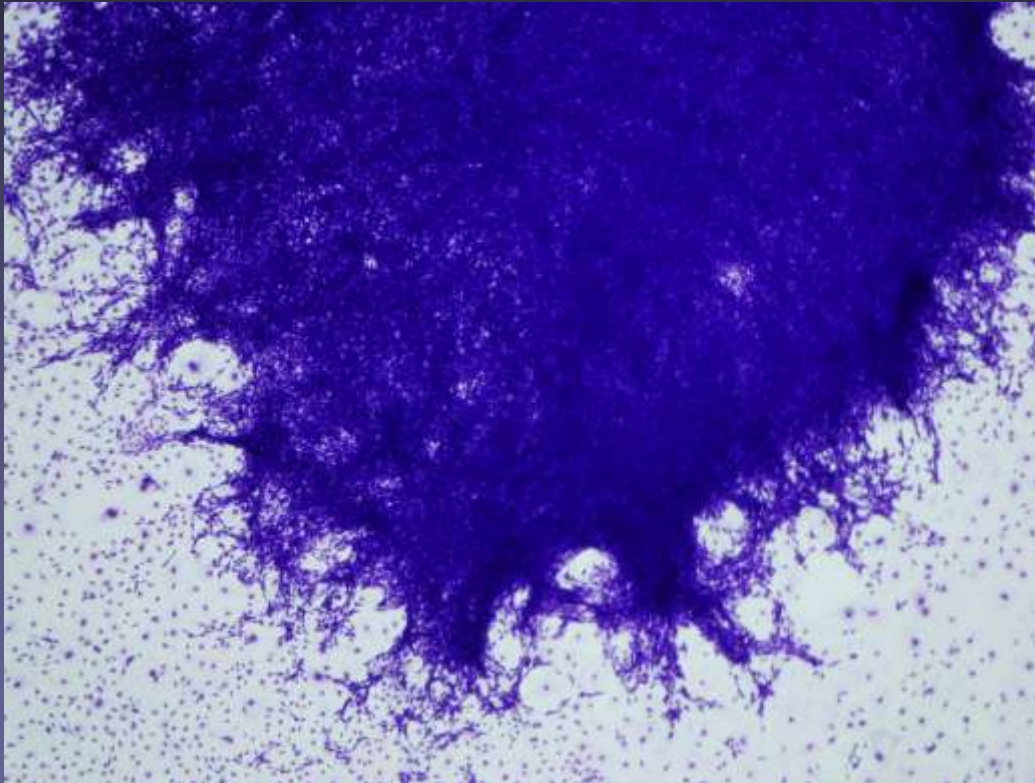
AXLR8-3

12 June 2012, Berlin

Outline of the Presentation

1. Bhas 42 cells
2. Methods of transformation assay
3. Validation studies and in-house study
4. H₂O₂ method

Focus (Transformed Colony) in BALB/c 3T3 Cells



Basophilic

Spindle-shaped

Criss-cross

Piling up

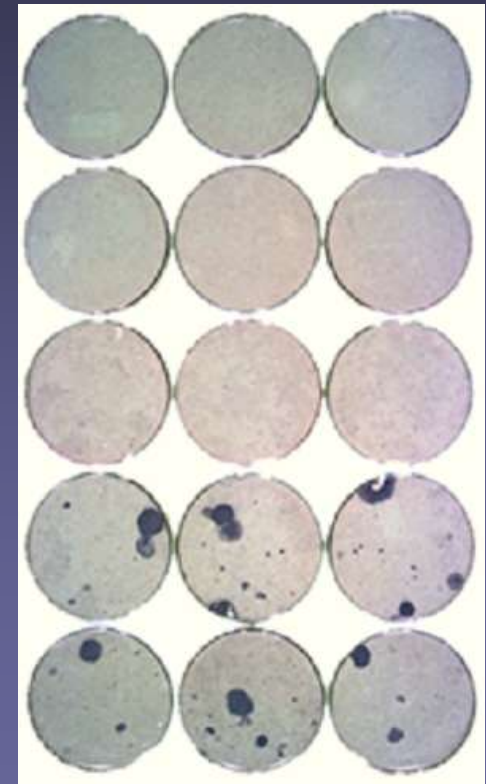
Invasive

Two Stage Transformation in BALB/c 3T3 Cells

0 1 2 3 4 5 6 wks

Initiator

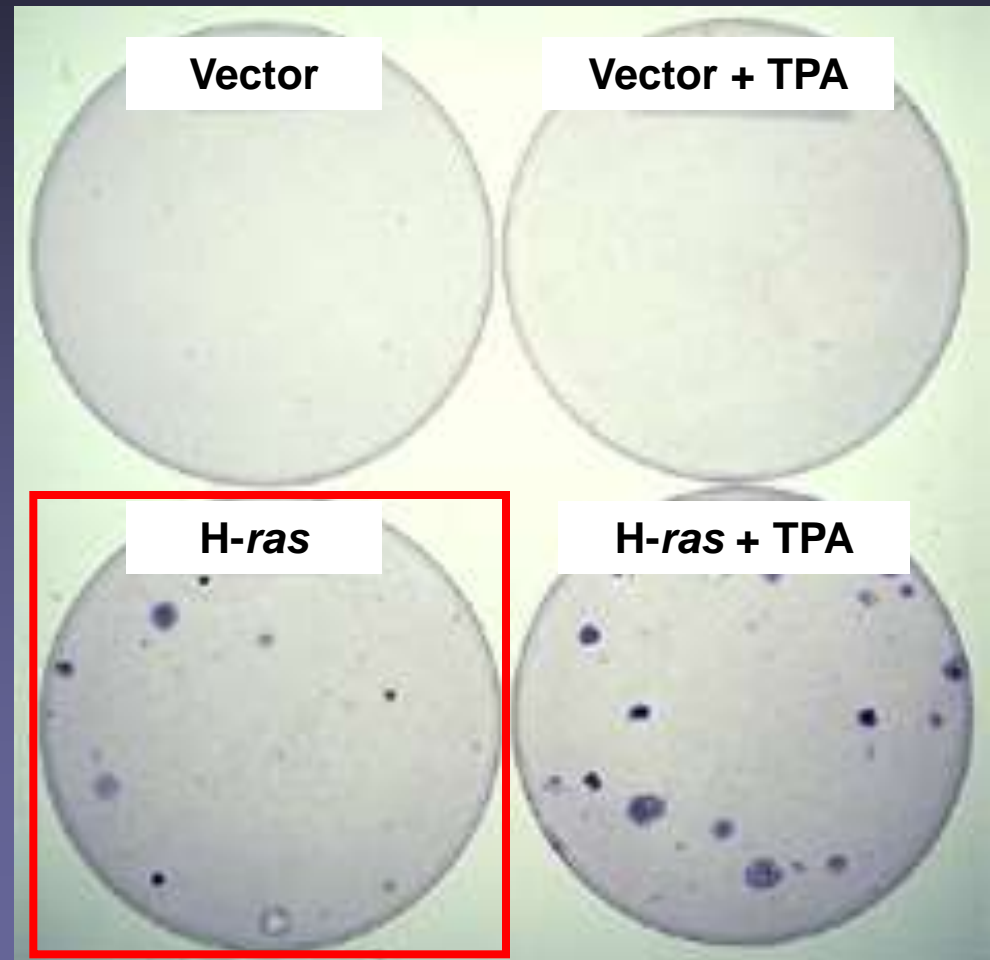
Promoter



Chemically Induced Initiation Can Be Replaced with *v-Ha-ras* in BALB/c 3T3 Cells

There are *v-Ha-ras* transfected normal phenotype cells, but they are changed morphologically by TPA.

A model of initiated cells.



Strategy for Isolation of Bhas 42 Cells

BALB/c 3T3 cells



Transfection of *v-Ha-ras*



Cloning of *v-Ha-ras* containing cells



Screening of non-transformed cells which are morphologically changed by TPA



Bhas 42 cells

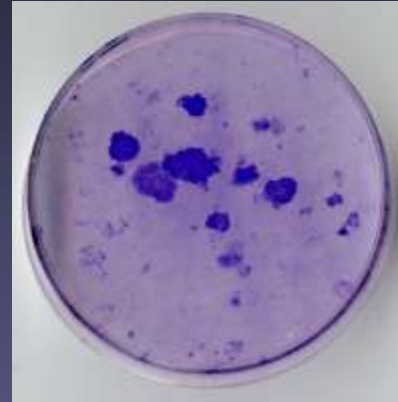
Bhas 42 Cells Are a Model of Initiated Cells

Control

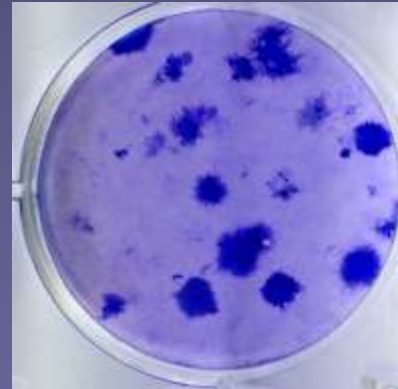
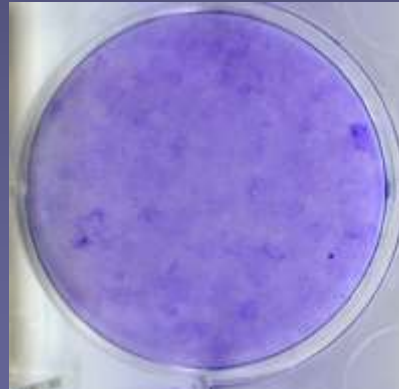
MCA

TPA

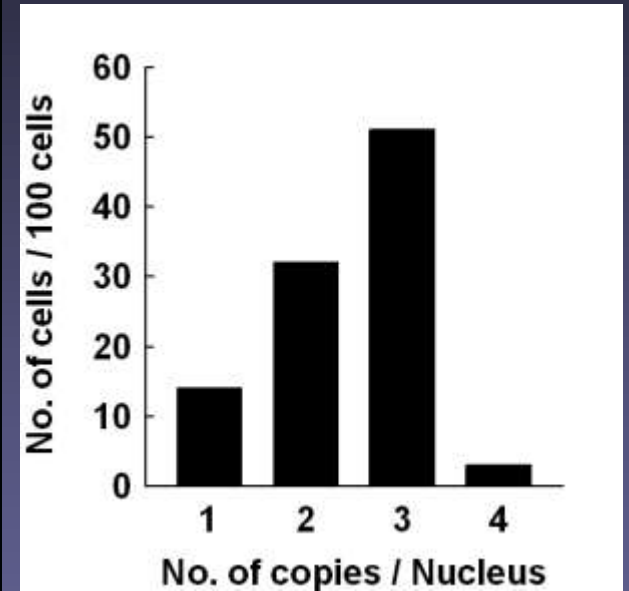
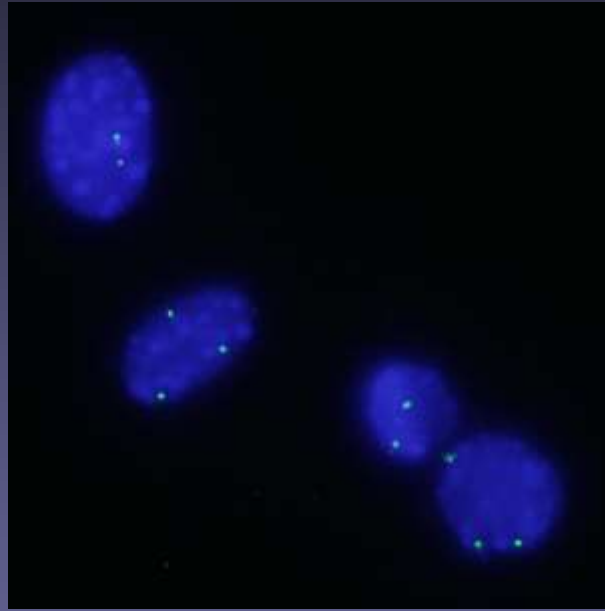
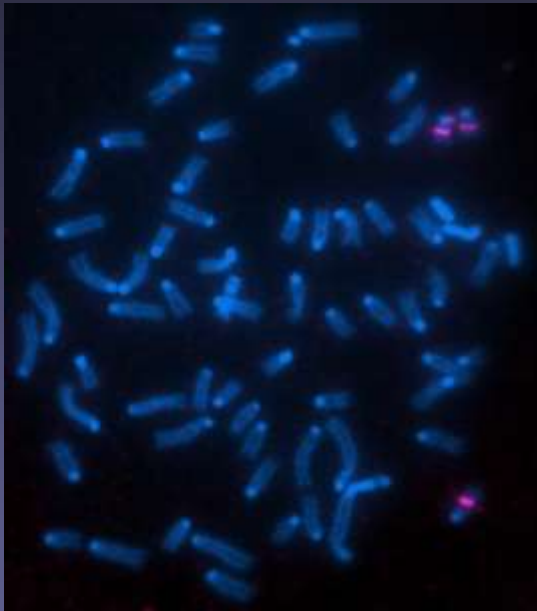
BALB/c 3T3



Bhas 42



FISH Analysis of v-Ha-ras in Bhas 42 Cells



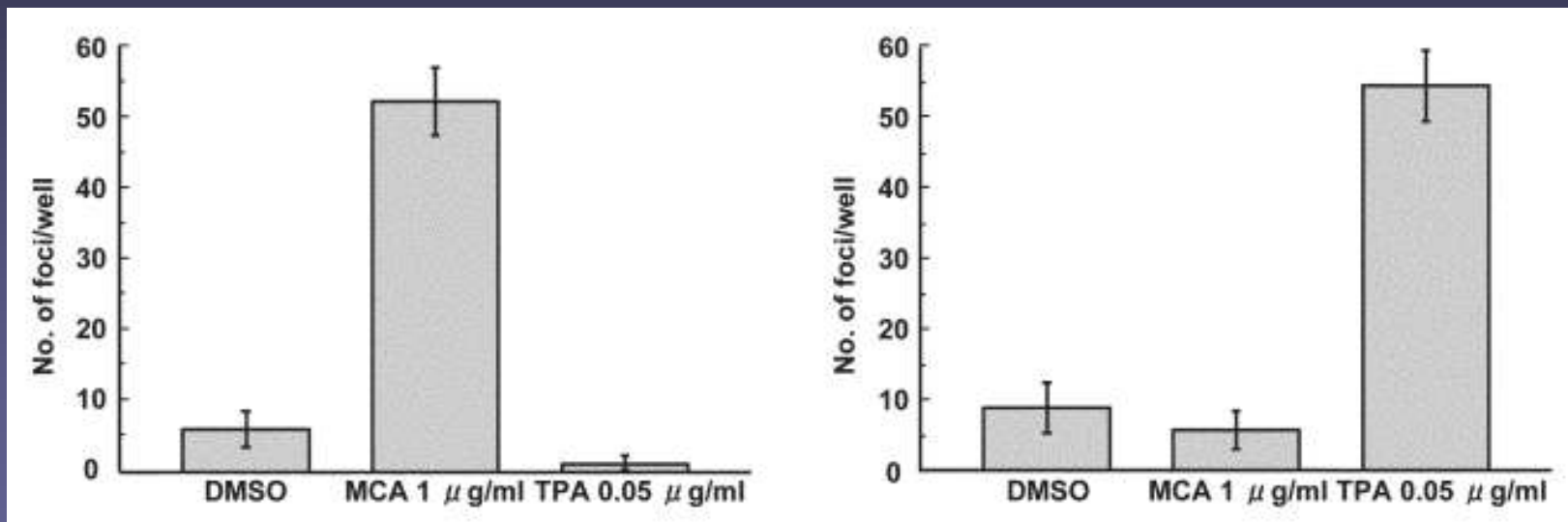
100% cells contain v-Ha-ras.

**Average =
2.4 copies / nucleus**

Initiators and Promoters Induce Transformation Depending on Cell State

Treated in growth phase

Treated in stationary phase

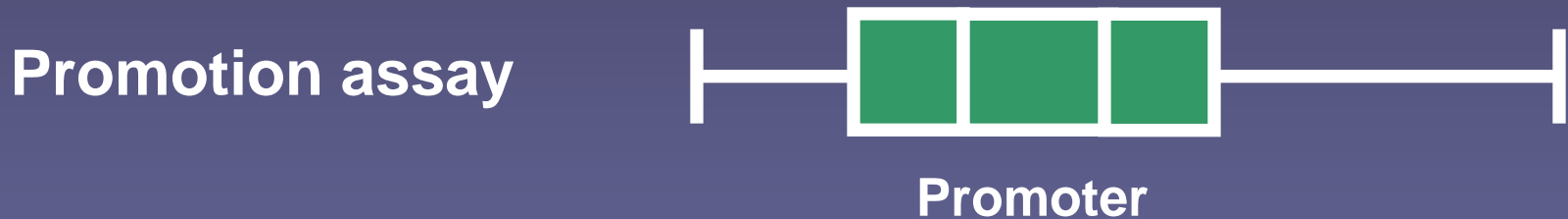


Sakai A. AATEX (2008) 14 special issue:367-373.

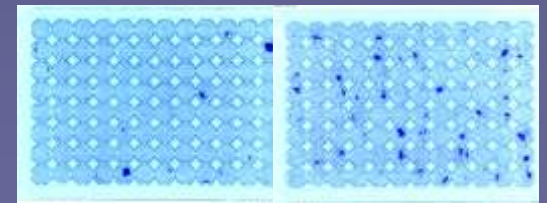
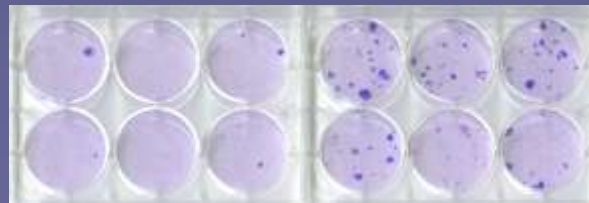
Protocol of Bhas 42 Cell Transformation Assay

Days 0 4 7 11 14 21

Medium change ↓ ↓ ↓ ↓



6 and 96-well plates can be used.



Overview of Validation Studies

Study No.	No. of labs	Plates	No.of chemicals / Study	No. of chemicals / Lab
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Pre-validation

1	6	6-well plate	9	3
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Validation

2	6	6-well plate	12	3
3 (Phase I)	4	96-well plate	7	4
3 (Phase II)	3	96-well plate	16	2

Total 23 chemicals
(excluding overlapping)

Results of Study No. 1 (6-Well Method)

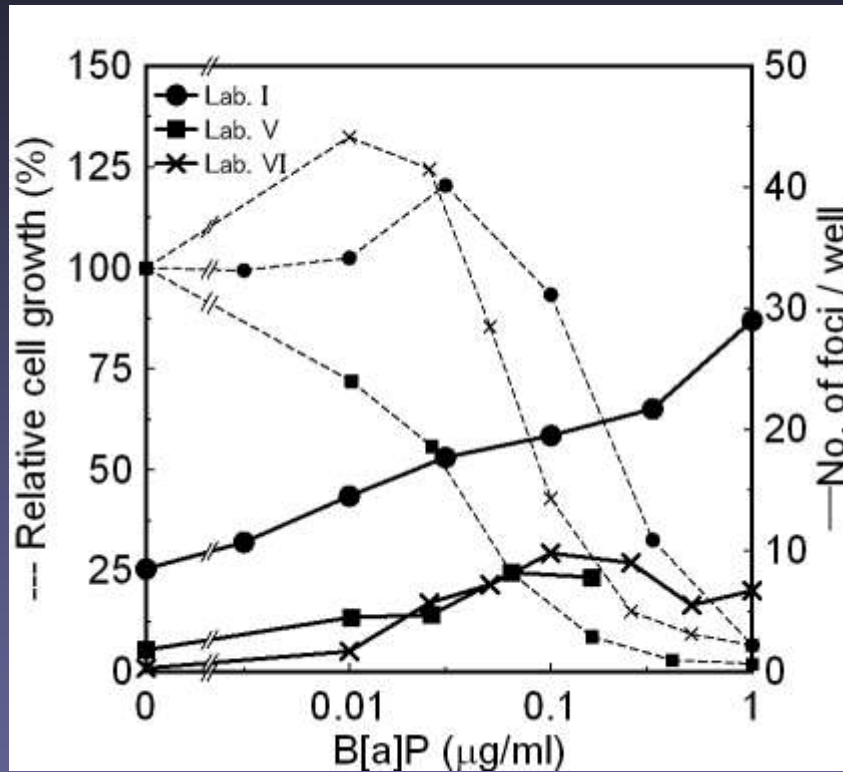
Labs	MNNG		B[a]P		Pyrene		B[a]A		Anthracene		Mezerein		LCA		Methapyrilene		Phorbol	
	I	P	I	P	I	P	I	P	I	P	I	P	I	P	I	P	I	P
I	+	-	+	-					-	-	-	+	-	+			-	-
II					+	+	+	+							-	+		
III	-	-							-	-			-	+				
IV											-	+					-	-
V			+	-	+	-	+	-							-	+		
VI	+	-	+	-	+	+	+	+	-	-	-	+	-	+	+	+	-	+/-
All over judgment	+	-	+	-	+	+	+	+	-	-	-	+	-	+	-	+	-	-
In vivo		+		+		-		+		-		+		+		+		-

I: initiation assay, P: promotion assay

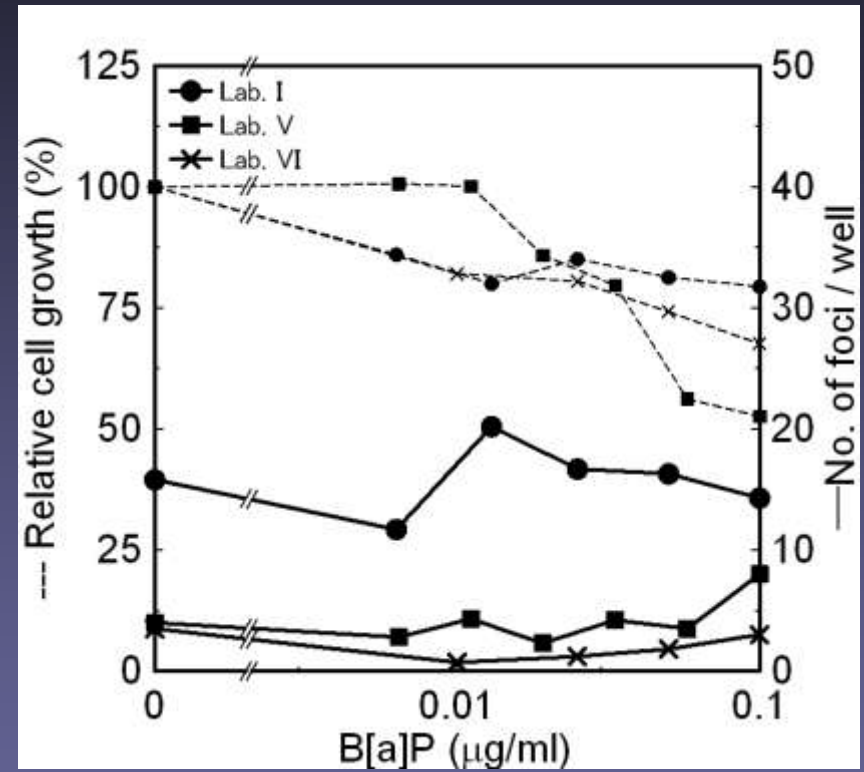
Reproducibility between labs: $49/53 = 92\%$

Concordance between the Bhas 42 assay and in vivo: $8/9 = 89\%$

Results of B[a]P in Study No. 1



Initiation assay



Promotion assay

Summary of Validation Studies

Study No.	No.of chemicals / Study	Reproducibility between labs	Concordance between Bhas 42 assay and in vivo
Pre-validation			
1	9	49/53 = 92%	8/9 = 89%
Validation			
2	12	49/67 = 93%	12/12 = 100%
3 (Phase I)	7	53/55 = 96%	6/7 = 86%
3 (Phase II)	16	64/64 = 100%	13/16 = 81%

Performances of Bhas 42 Assay: vs In Vivo

		In vivo carcinogenicity	
		Carcinogen	Non-carcinogen
Bhas 42 assay	Positive	38	6
	Negative	14	31

89 chemicals in total

Concordance $78\% = (38 + 31) / (38 + 14 + 6 + 31) \times 100$

Sensitivity $73\% = 38 / (38 + 14) \times 100$

Specificity $84\% = 31 / (6 + 31) \times 100$

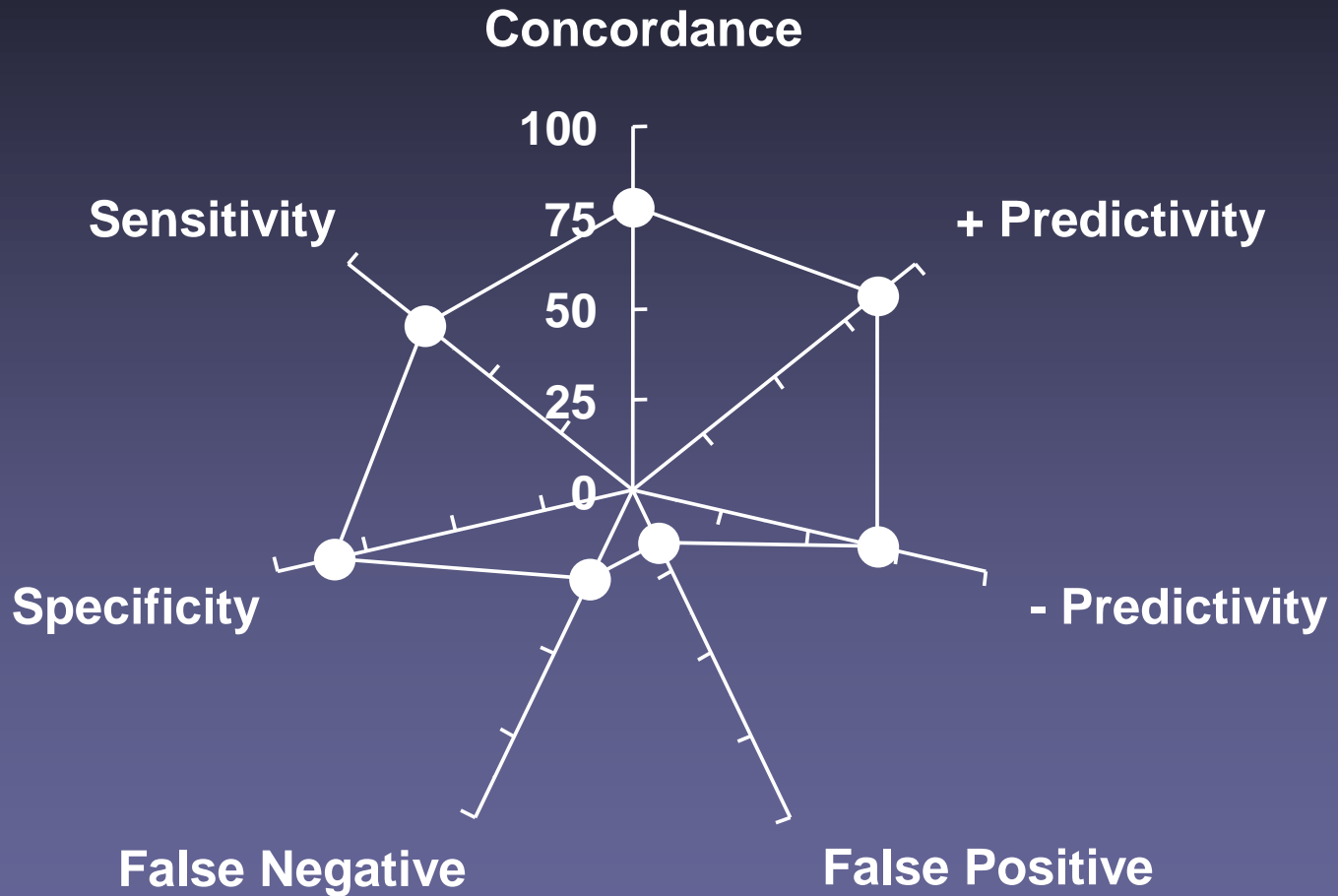
Positive predictivity $86\% = 38 / (38 + 6) \times 100$

Negative predictivity $69\% = 31 / (14 + 31) \times 100$

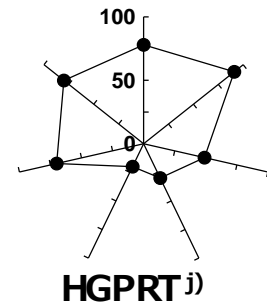
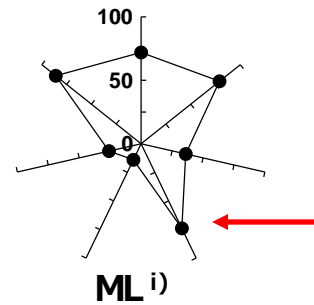
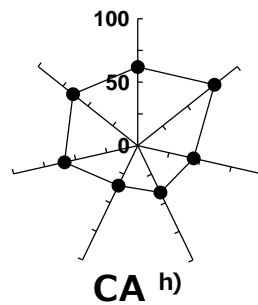
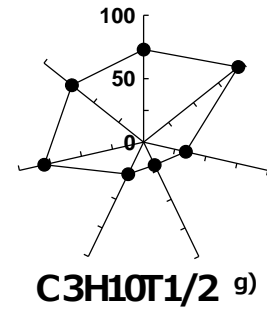
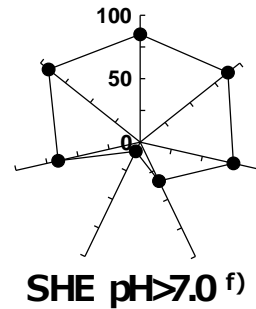
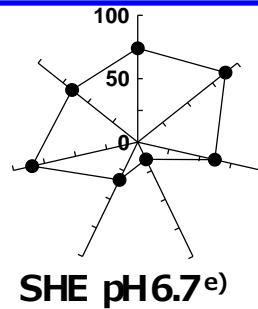
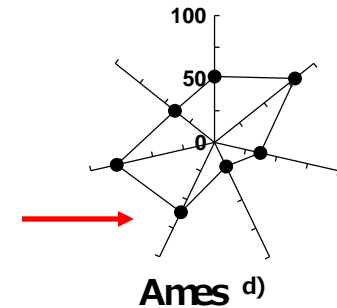
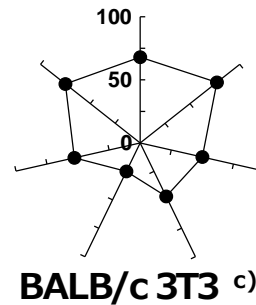
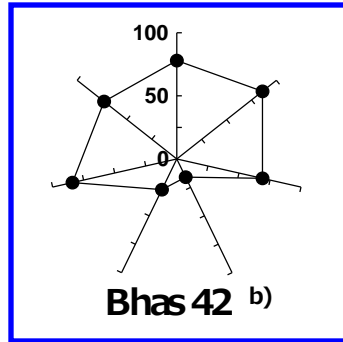
False positive $16\% = 6 / (6 + 31) \times 100$

False negative $27\% = 14 / (38 + 14) \times 100$

Performances of Bhas 42 Assay: vs In Vivo



Performances of In Vitro Genotoxicity Tests

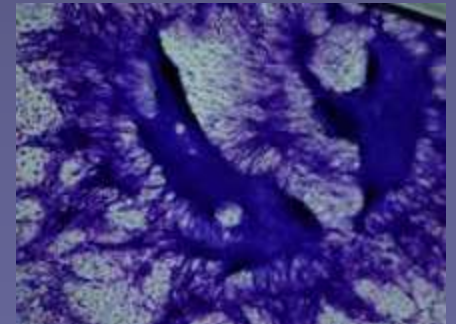
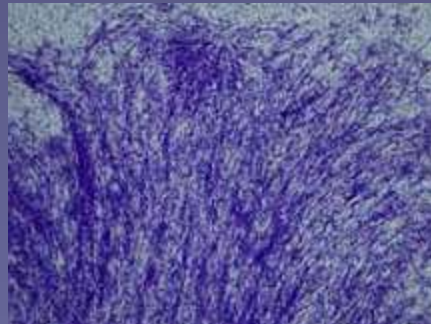
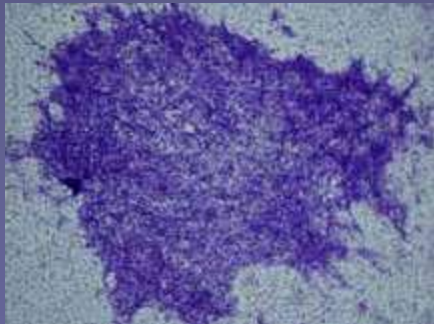
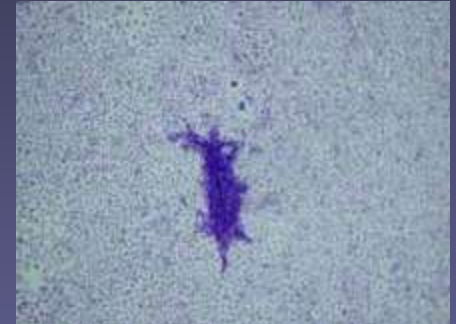
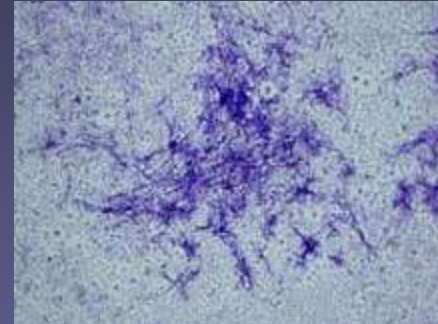
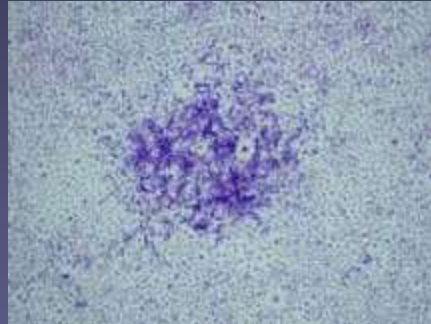
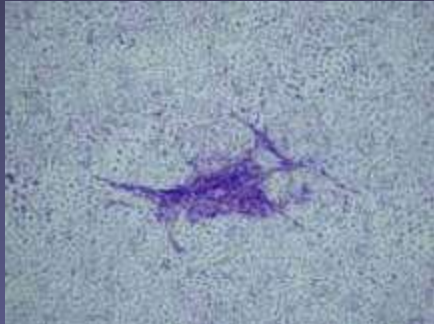


All data except Bhas 42 cells obtained from DRP31 (2007).

Various Morphologies of Foci in Bhas 42 Cells

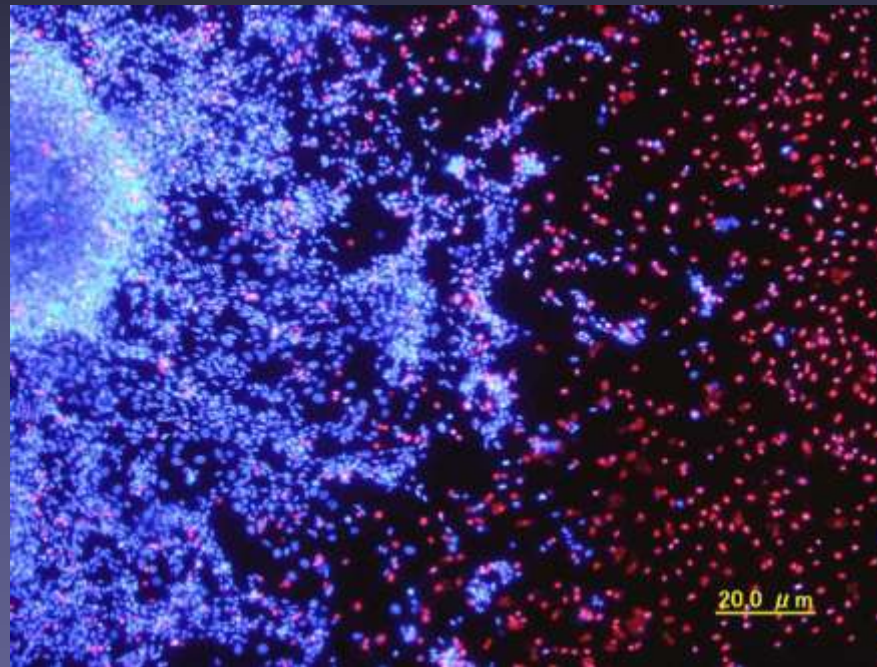


- Subjective judgement
- Time consuming observation



Selective Induction of Necrosis by H₂O₂ in Bhas 42 Normal Cells

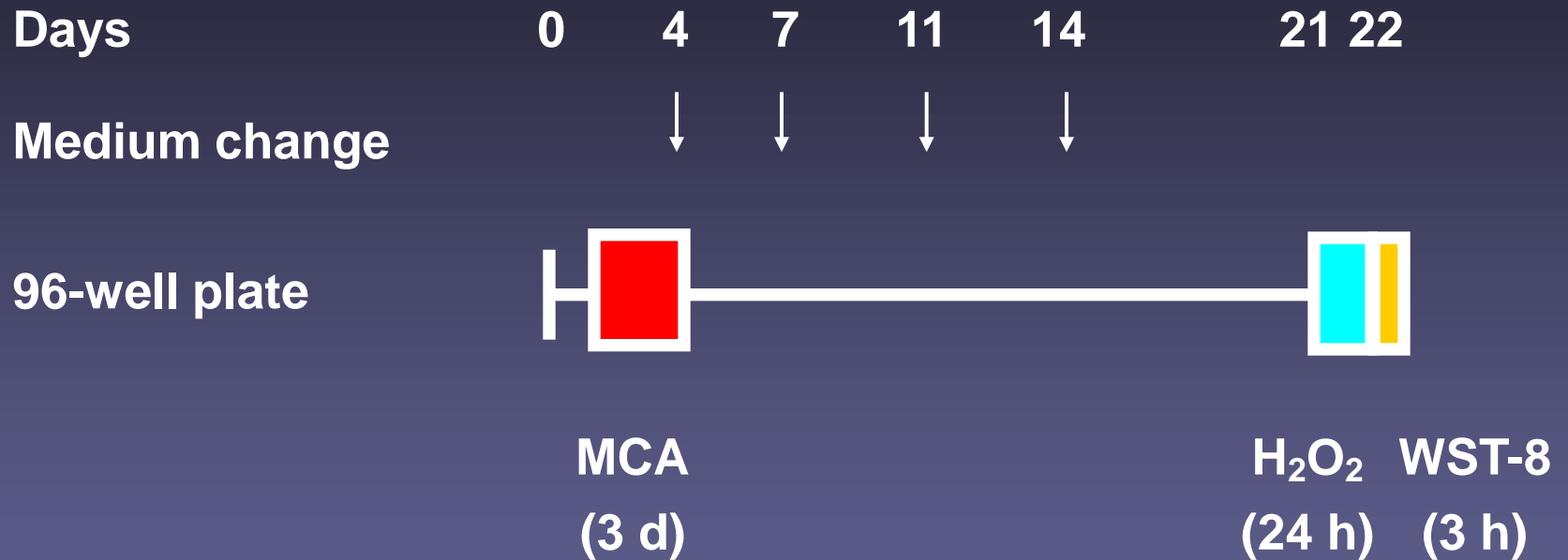
Treatment with H₂O₂ (0.0015%) for 24 h



Living transformed cells
(Hoechst 33342)

Killed normal cells
(Ethidium homodimer III)

Protocol of H₂O₂ Method



H₂O₂: 0.0015%

WST-8: 5%, OD at 450 nm

Concordance between Wells Colored by WST-8 and Wells with Transformed Foci

DMSO 0.1%

MCA 1 $\mu\text{g/mL}$

H_2O_2 + WST-8



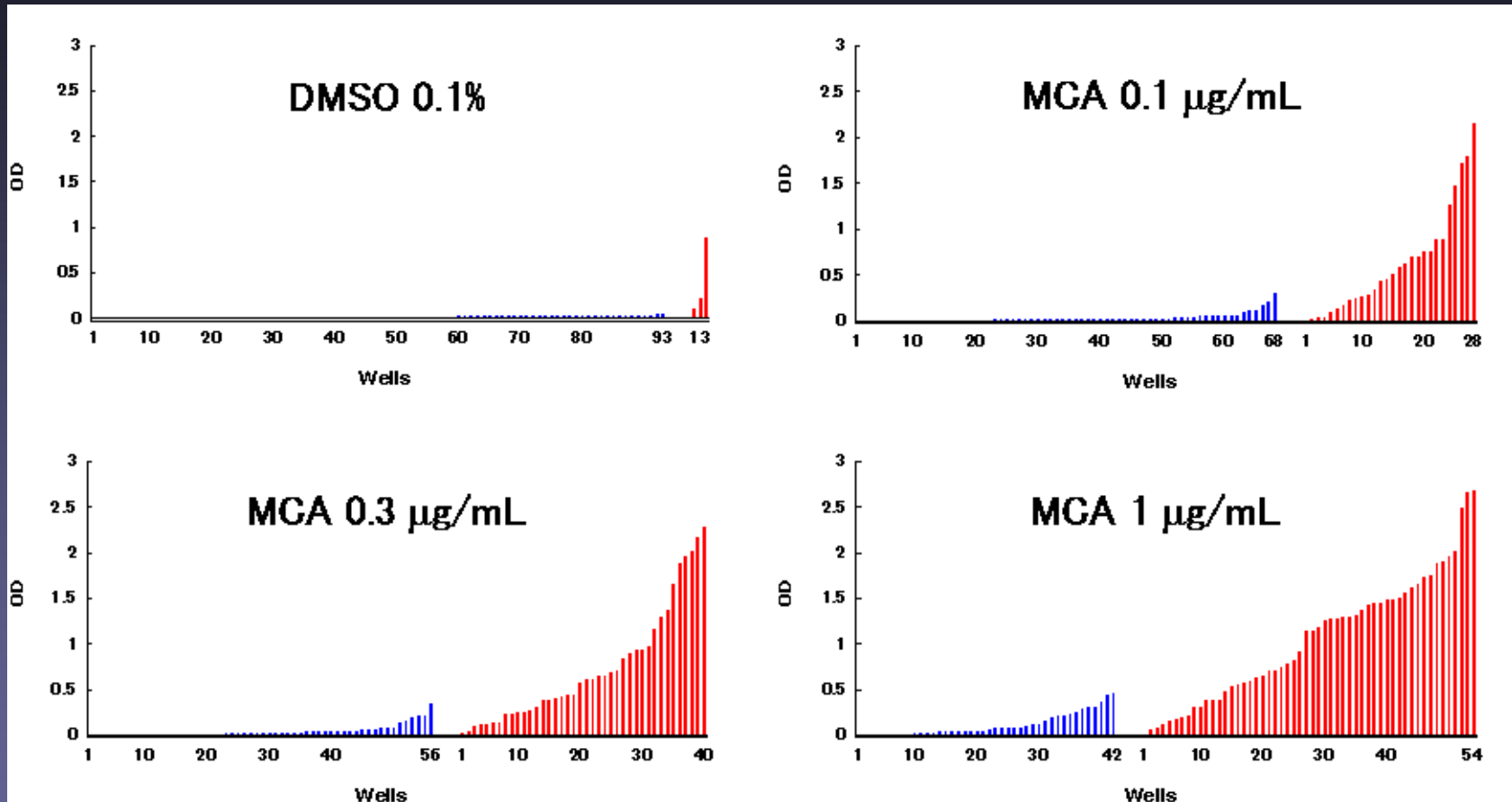
Fixation



Giemsa staining



Dose-Dependent Increase of OD Values by MCA





■ : wells without transformed foci, ■ : wells with transformed foci.

Advantages of H₂O₂ Method

	Observation method	H ₂ O ₂ method
Equipment	Microscope	Plate reader
Quantification	Subjective	Objective
Time	Time consuming observation	Short time measuring
Labor	Fixation, staining, washing, drying	Just adding
Automatic system	Not easy	Easy



Bhas 42 Assay vs Animal Test

	Transformation	Carcinogenicity
	 <p>Bhas 42 cells</p>	 <p>Animals (Rats, Mice)</p>
Test Period	< 1 month	2-3 years
Cost	< Ten thousand \$	> Two million \$

Conclusion

1. Bhas 42 assay is reproducible.
2. Bhas 42 assay can predict carcinogenicity well.
3. H_2O_2 method is useful to quantify transformation frequency.

Situation of Bhas 42 Assay to Apply OECD Guideline

2006

2010

2012



Start

Submit SPSF

Submit validation report

Submit background report

Acknowledgements

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BioReliance (USA)

Pant

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Poth

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Bioassay Research Center (Japan)

Asakura

Ina Research (Japan)

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Mitsubishi Chemical Safety Institute (Japan)

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