111年度動物用生物藥品產業人員教育訓練

(1)組織培養疫苗研發現況

(2)動物用疫苗製程改良

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組織培養疫苗研發現況

- 提升疫苗廠現有病毒性疫苗產品生產效能： 增加病毒力價。
- 開發新型疫苗：量產過去無法以細胞培養量產之抗原 。
- 改變病毒疫苗製程，降低汙染： 細胞培養取代活體動物及雞胚胎。

細胞暨病毒技術

病毒性疫苗

抗原含量

限制

Live vaccine:$10^{2-5}TCID_{50}$

Inactive vaccine:$10^{5-7}TCID_{50}$

SPF雞胚不易取得

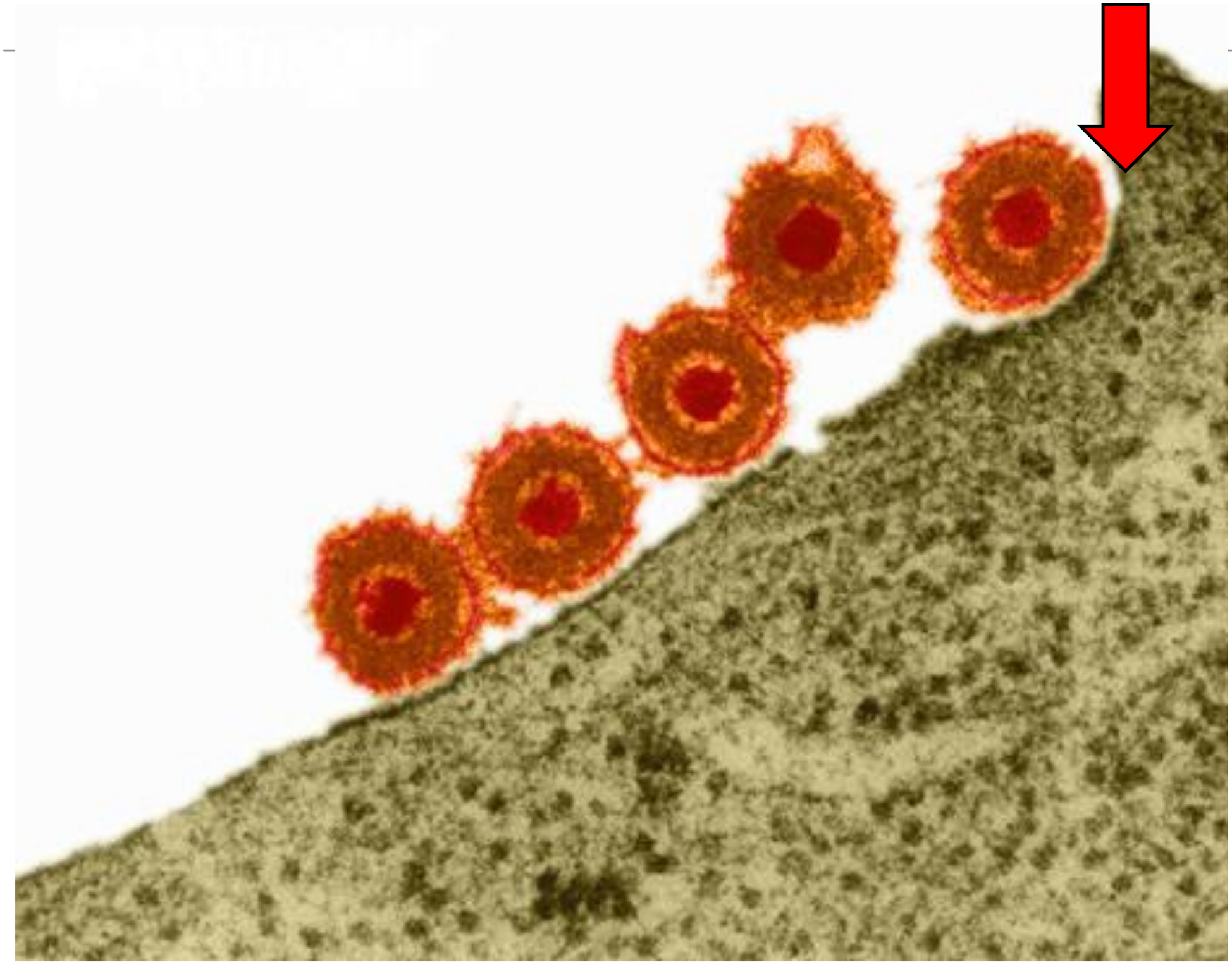
低抗體蛋力價不穩定

許多抗原無法在雞胚或細胞株增殖出有經濟價值力價

找尋可用以生產抗原之細胞株

提升細胞株抗原產量

1.細胞必須要有病毒的接受體



2.病毒必須要能克服細胞干擾素系統

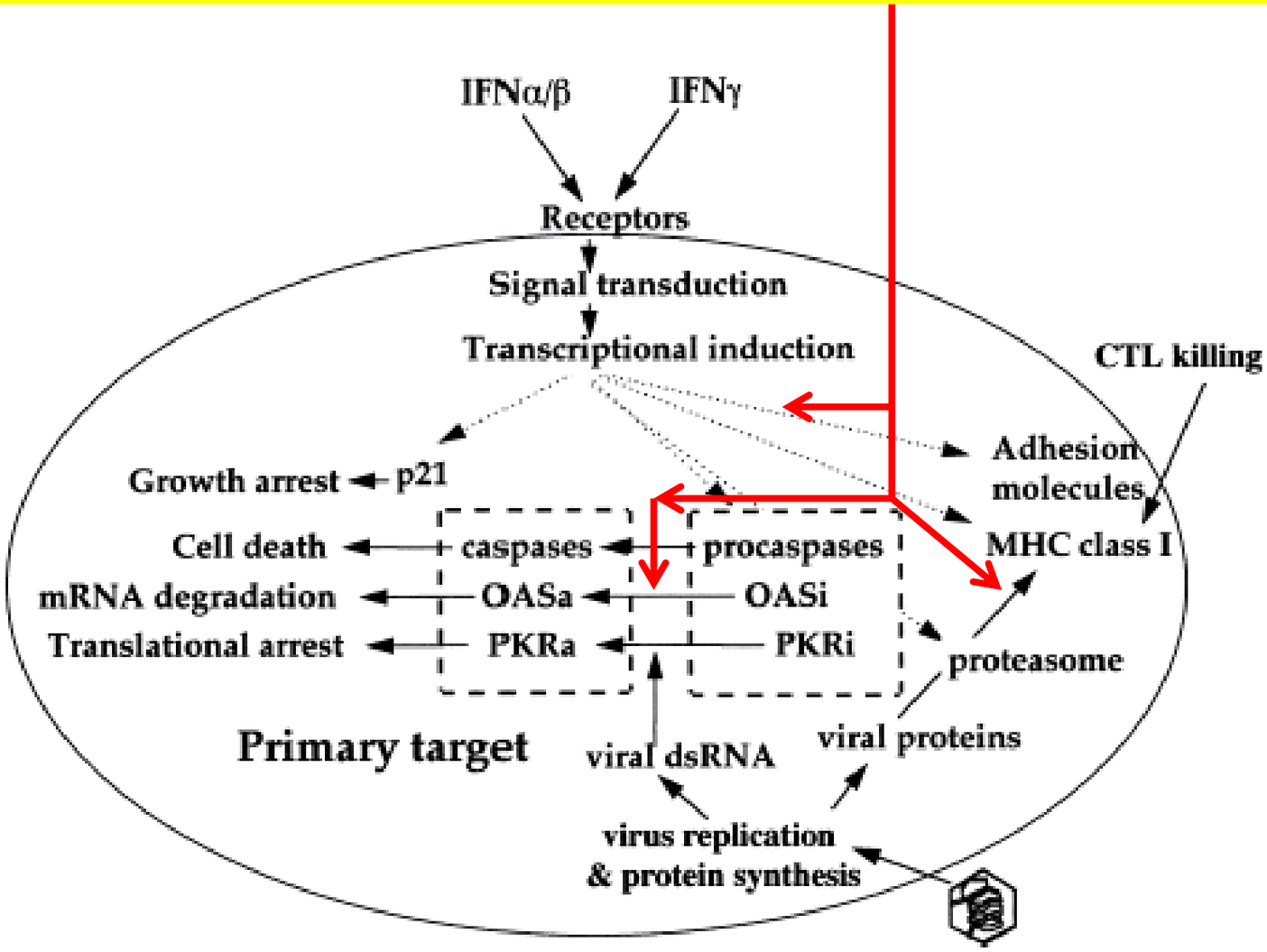
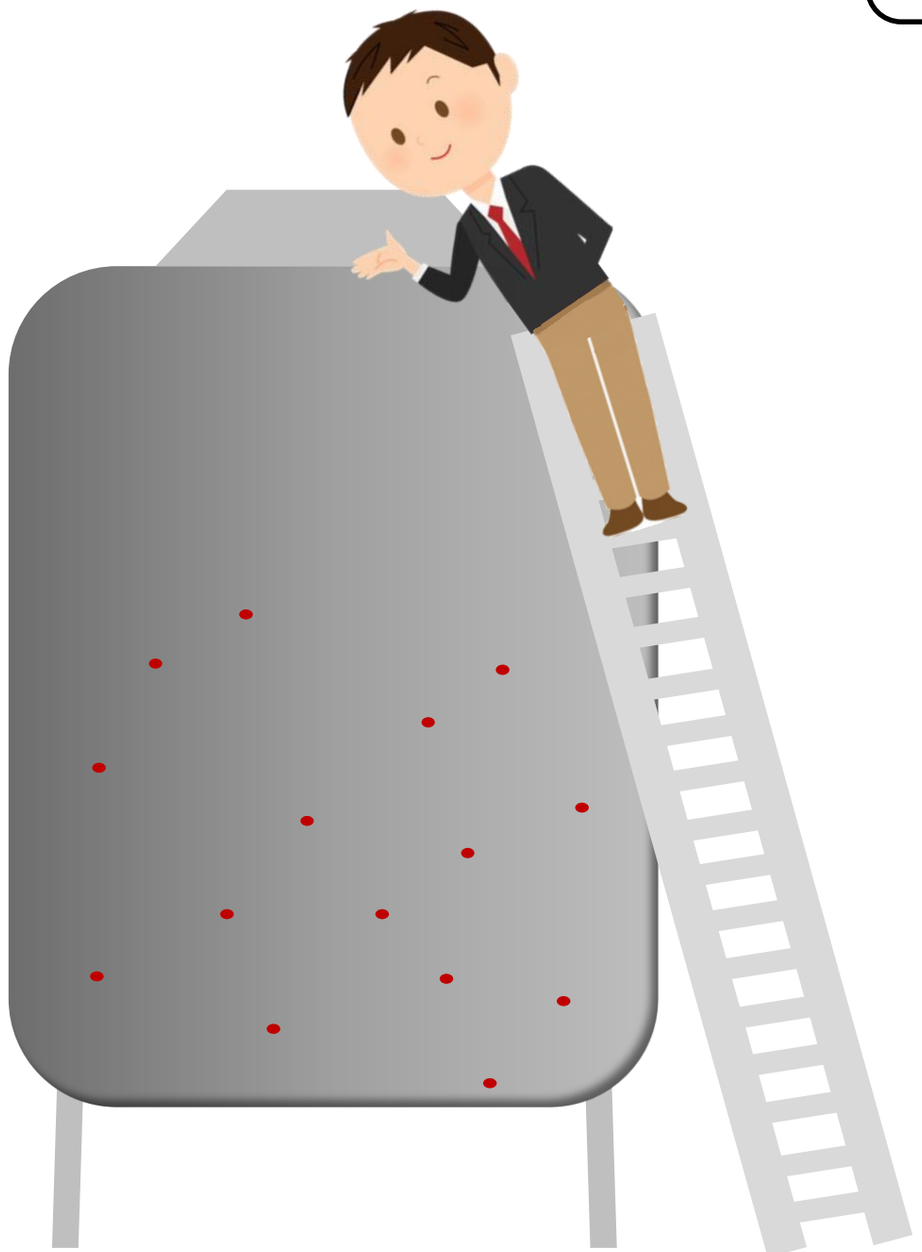


Fig. 1. The biological properties of α/β and γ IFNs. IFNs α/β and γ bind to specific surface receptors on primary target cells and induce the transcription of a variety of genes that mount an antiviral response. It is characteristic of these gene products that they often depend upon viral dsRNA as a co-factor in order to ensure that they are only active under conditions of infection. Thus, PKR and 2′–5′ oligoadenylate synthetase (OAS) are synthesized as inactive precursors (PKRi and OASi, respectively) and are activated by dsRNA (PKRa and OASa, respectively). Once activated, these gene products shut down translation. IFNs can also induce the synthesis of gene products that arrest the cell cycle (e.g. p21, an inhibitor of G_1/S phase-specific cyclin-dependent kinases), thus blocking virus replication, or induce a pro-apoptotic state (e.g. procaspases). Finally, IFNs can induce the synthesis of proteins that are involved in the processing and presentation of virus proteins to $CD8^+$ cytotoxic T lymphocytes (CTLs) (e.g. MHC class I proteins, components of the proteasome and peptide transporter molecules). Both types of IFN also have profound immunomodulatory effects that differ between types, and these are discussed in the text.

(Goodbourn *et al*., 2000)

高密度生產

量產



高密度生產

Engineered and clonally selected cell lines

for higher virus titer, better antigenicity, and lower costs

1.Cell/Virus clone selection

2.Expression of virus receptors in cells

3.Blocking cells’ antiviral response

4. Immortalization of primary cells

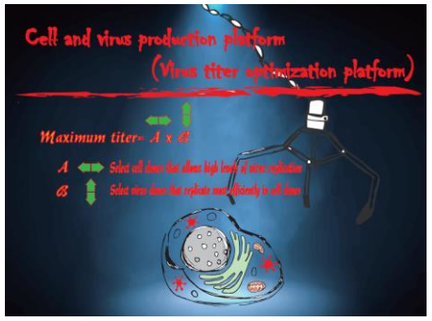
5. Serum-free suspension cell culture

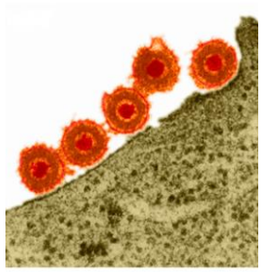
Engineered and clonally selected cell lines

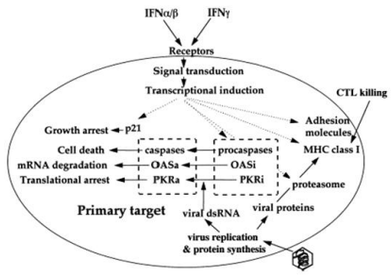
for higher virus titer, better antigenicity, and lower costs

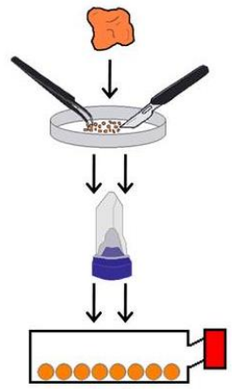
Cells

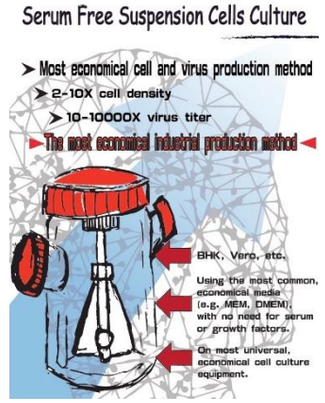
- Cell/Virus clone selection
- Expression of virus receptors in cells
- Blocking cells' antiviral response
- Immortalization of primary cells
- Serum-free suspension cell culture





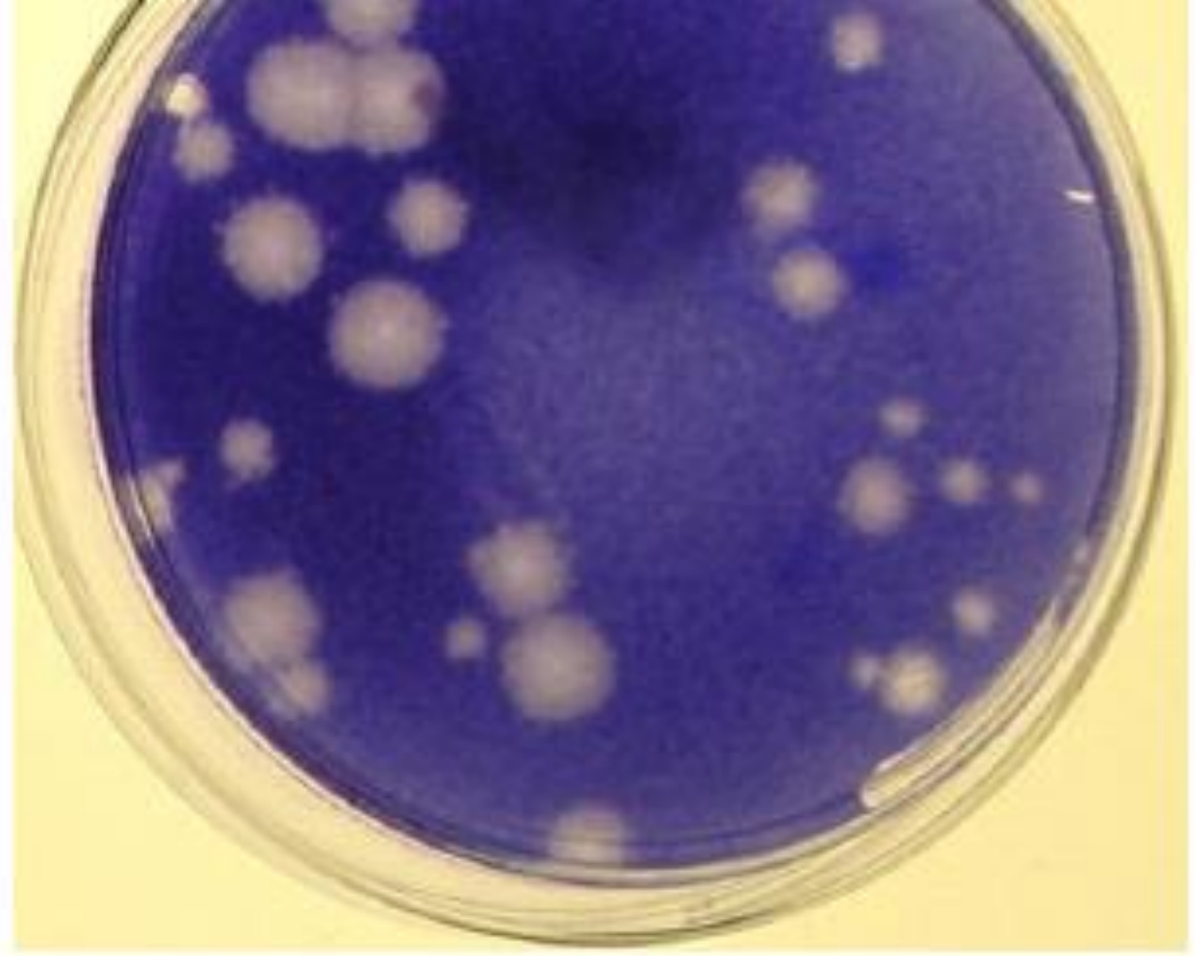


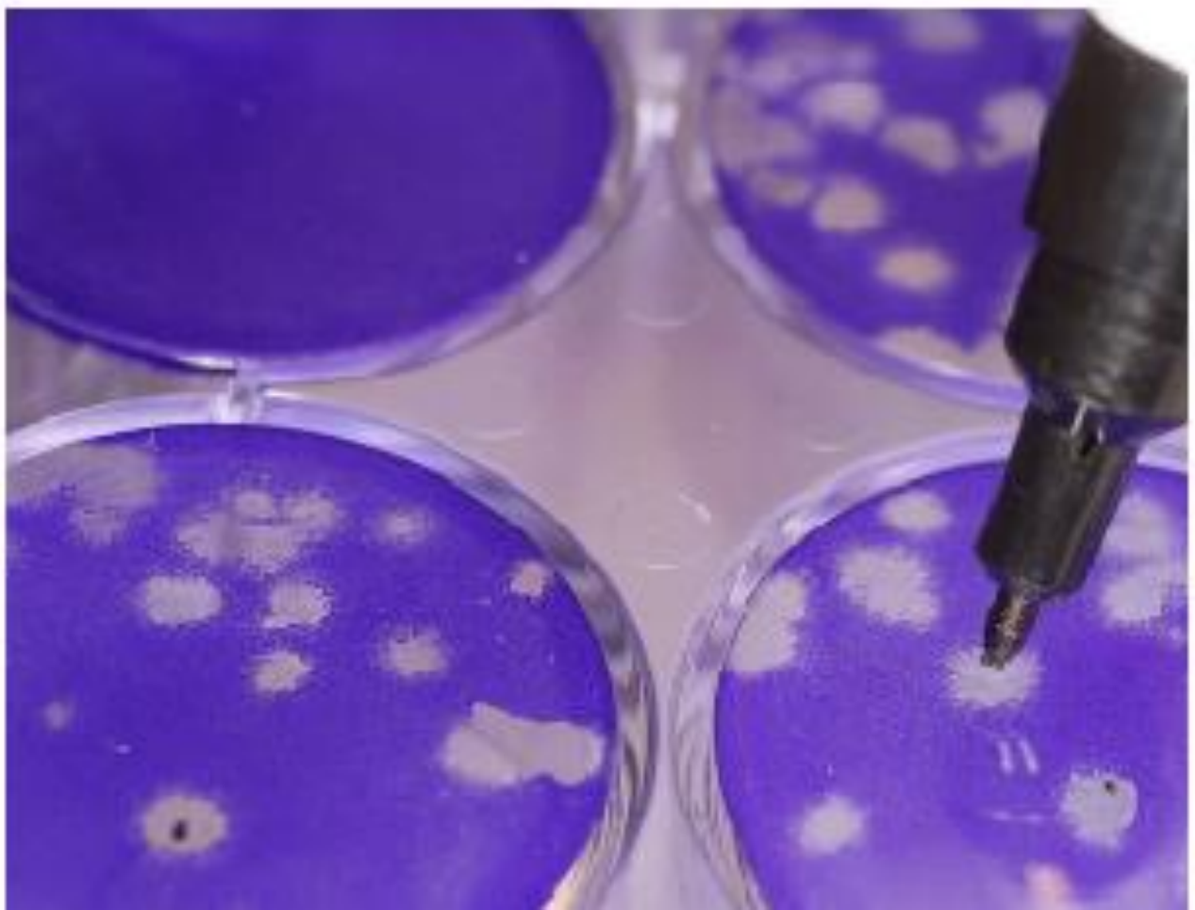




1

Cell/Virus clone selection





種毒/種細胞株選殖技術

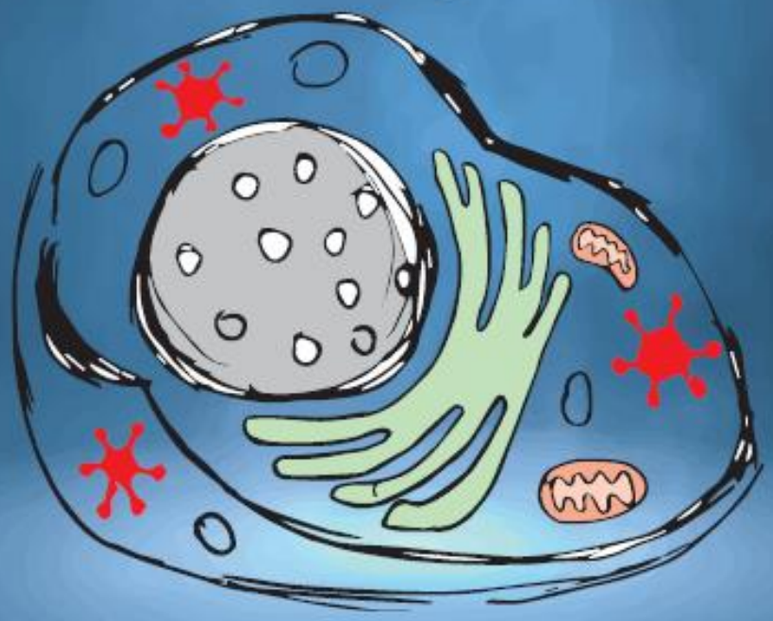
每一顆細胞/病毒都是獨立的個體

Cell and virus production platform
(Virus titer optimization platform)

Maximum titer= A x B

A ⟷ Select cell clones that allows high levels of virus replication

B ↕ Select virus clones that replicate most efficiently in cell clones



病毒馴化

病毒

細胞株

劇烈突變

死亡

不易存活/
失去抗原性?

病毒

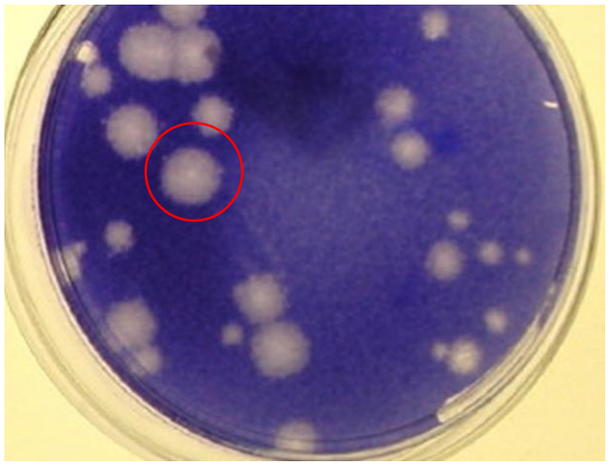
基因工程細胞

突變

死亡

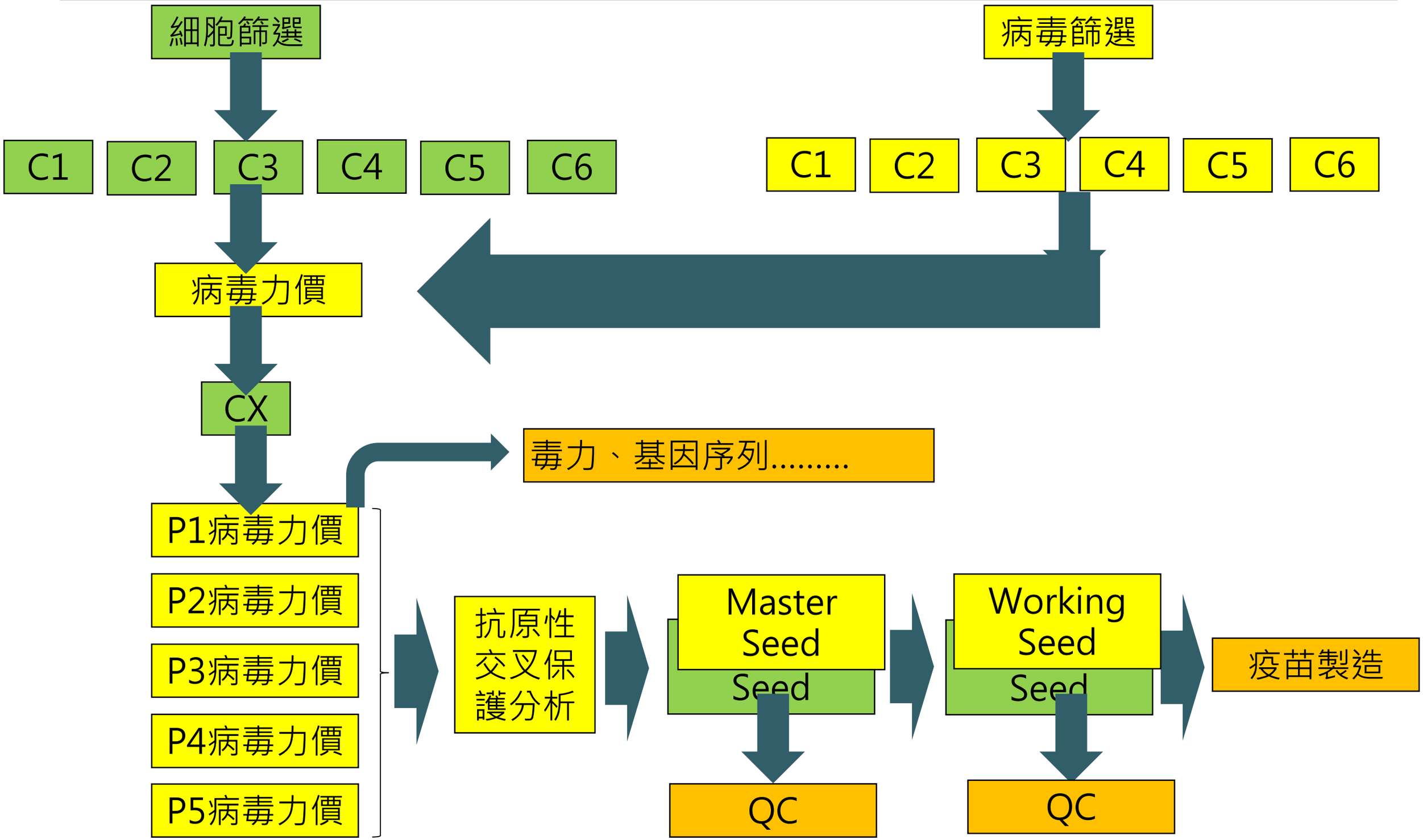
容易存活/
保留抗原性

生產用細胞株

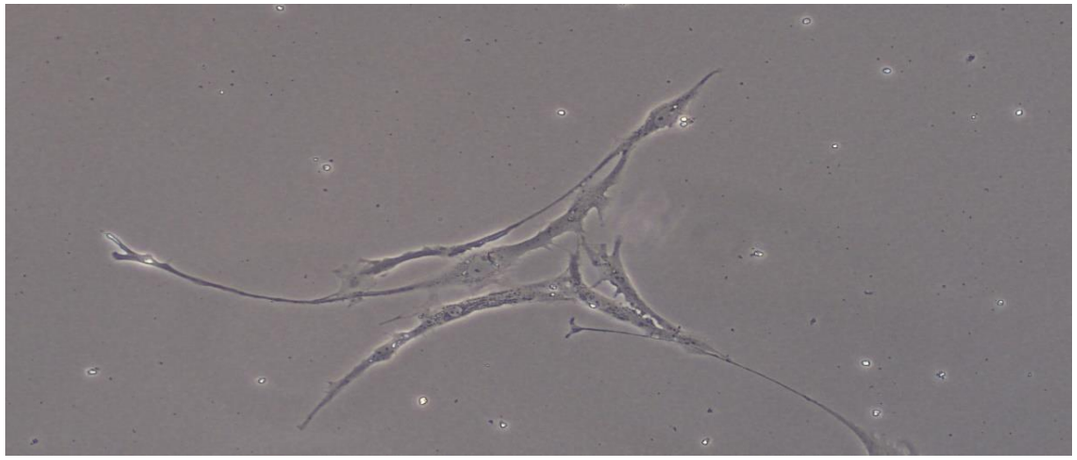


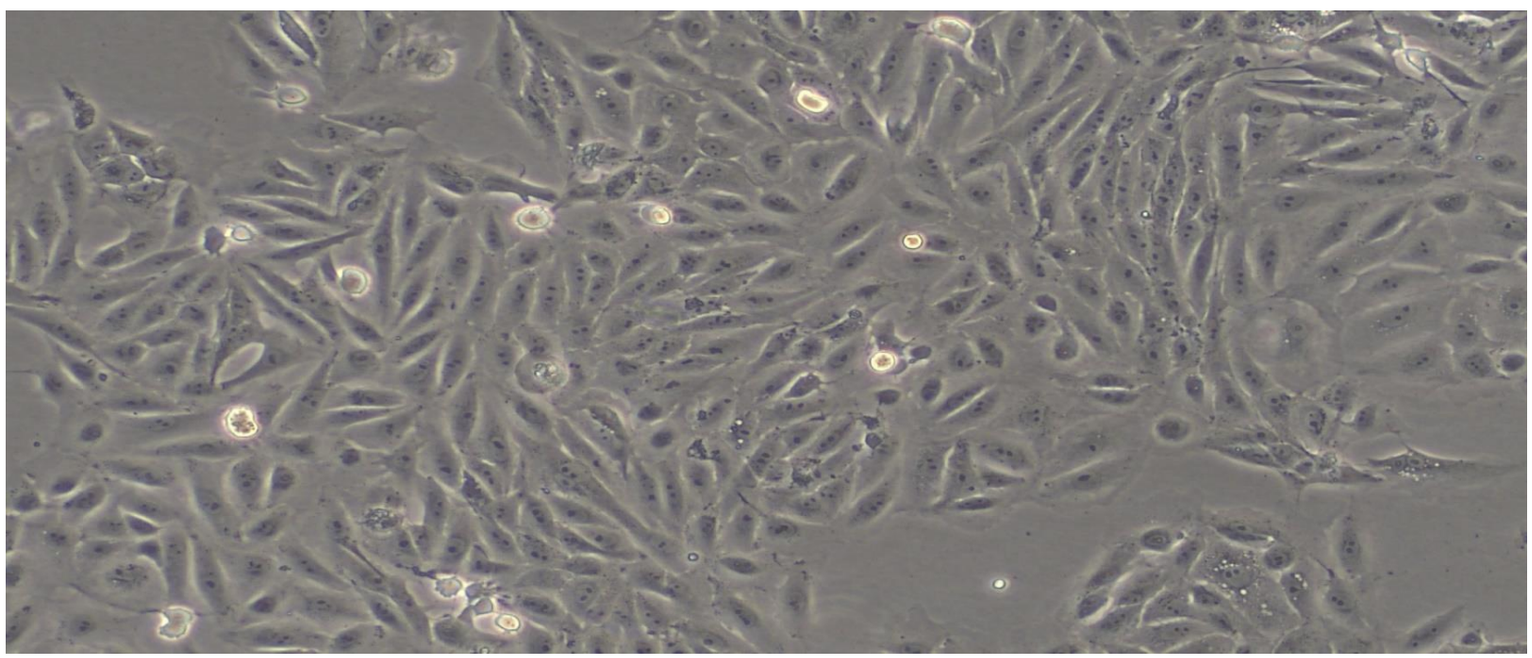
選殖

連續繼代


細胞篩選
病毒篩選
C1
C2
C3
C4
C5
C6
C1
C2
C3
C4
C5
C6
病毒力價
CX
毒力、基因序列.....
P1病毒力價
P2病毒力價
P3病毒力價
P4病毒力價
P5病毒力價
抗原性
交叉保
護分析
Master
Seed
Seed
Working
Seed
Seed
疫苗製造
QC
QC

細胞選殖

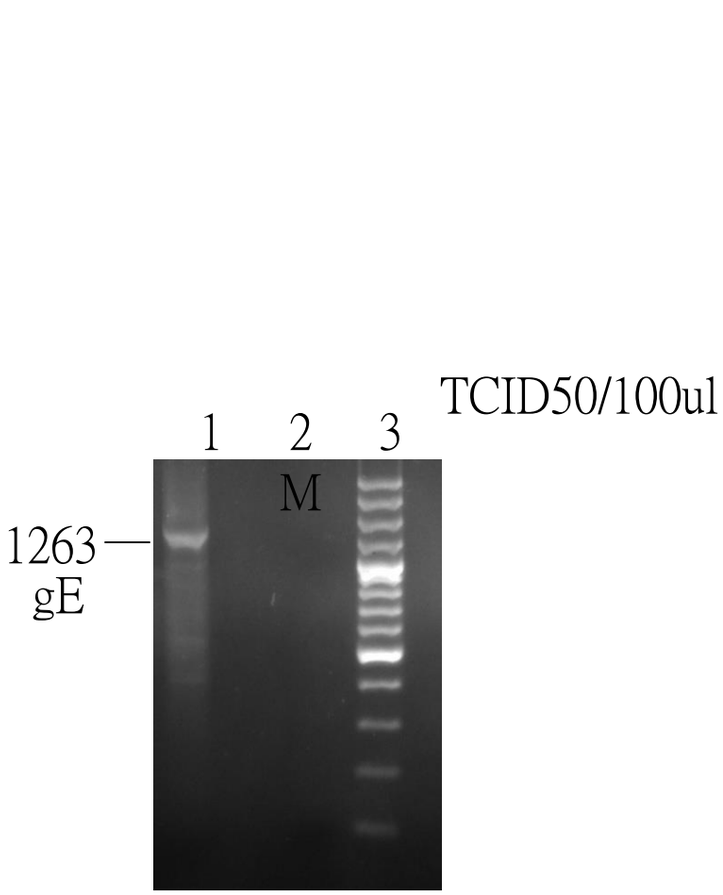




PK-207/ Porcine Kidney Cell

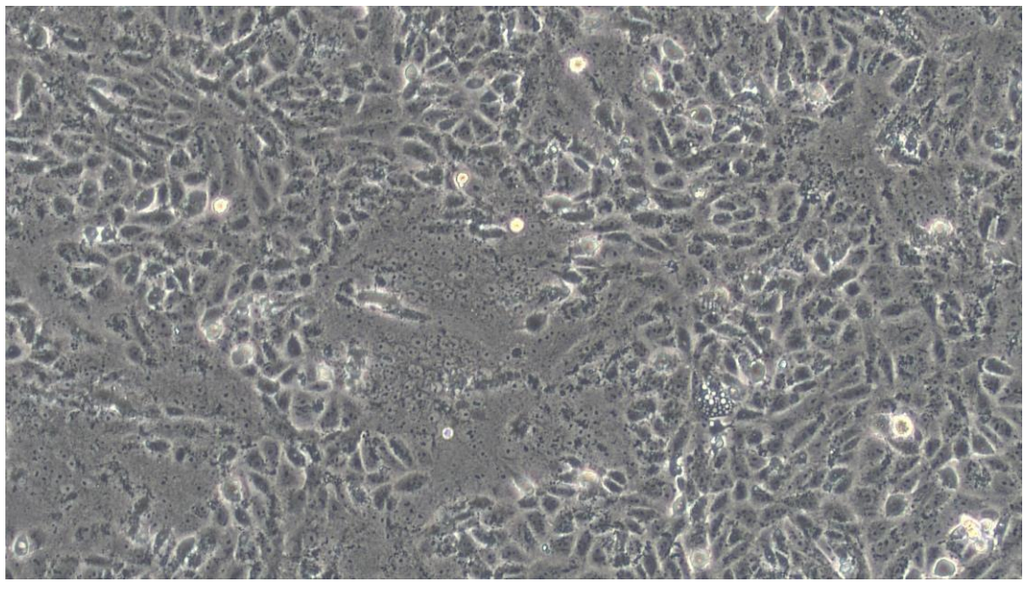
PR (Bartha K61)

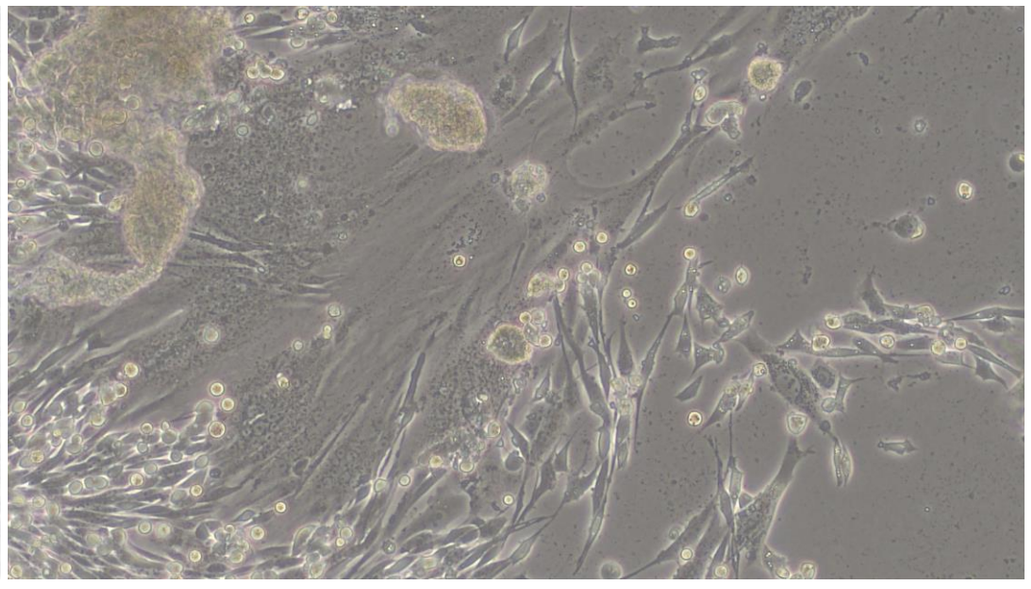
New PR (HN1401)



Higher titer of PEDV through viral clonal selection

	P0	P1	P2	P3	P3C3	P4C3	P5C3	P6C3	P7C3	P8C3	P9C3	P10C3
Cells	Vero	Vero	Vero	Vero	Vero	Vero	Vero	Vero	Vero	Vero	Vero	Vero
Ct	14.18	10.05	10.3	6.02	7.79	6.55	ND	ND	ND	ND	ND	ND
TCID50 (log10)/100ul	6.33	8	8	10.5	10.33	10.33	9	10	9	10	10	9





抗原性分析

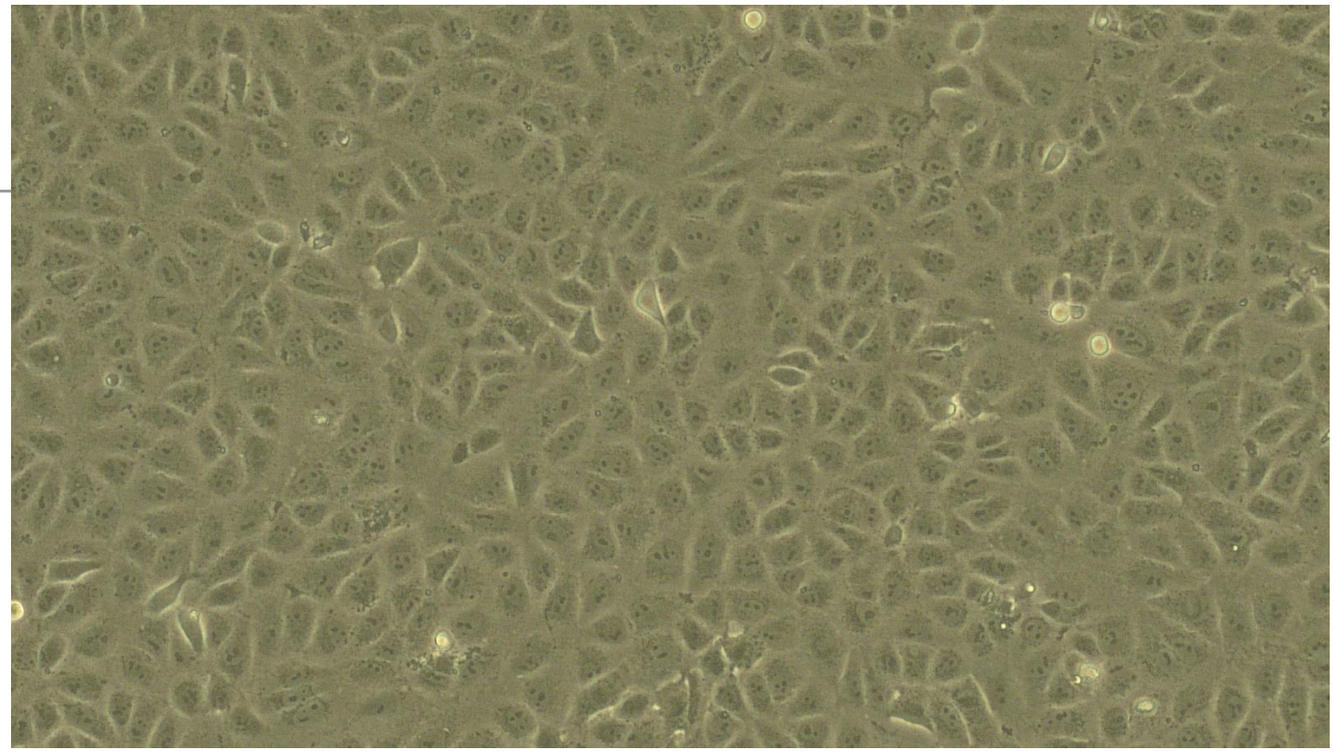
		Anti-Serum(NT log2)			
		PED-P0*	PED-P4C3*	PED-P8*	PR**
Antigen	PED-P0	5	6	5	0
	PED-P4C3	5	6	5	0
	PED-P8C3	5	6	5	0
	PR	0	0	0	4

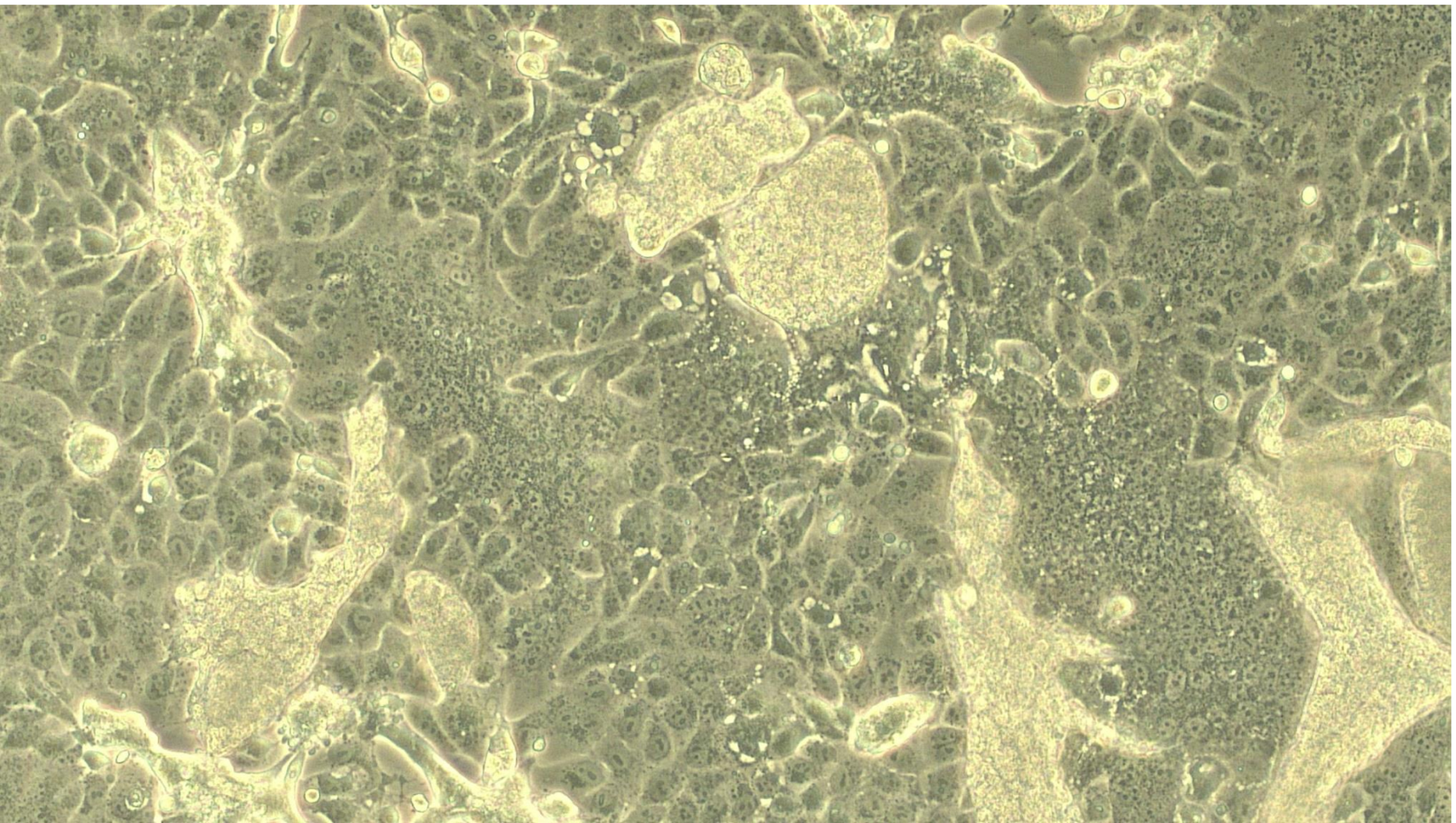
*小鼠,**猪

		Anti-Serum r-vaule%			
		PED-P0*	PED-P4C3*	PED-P8*	PR**
Antigen	PED-P0	100			
	PED-P4C3	100	100		
	PED-P8C3	100	100	100	
	PR	0	0	0	100

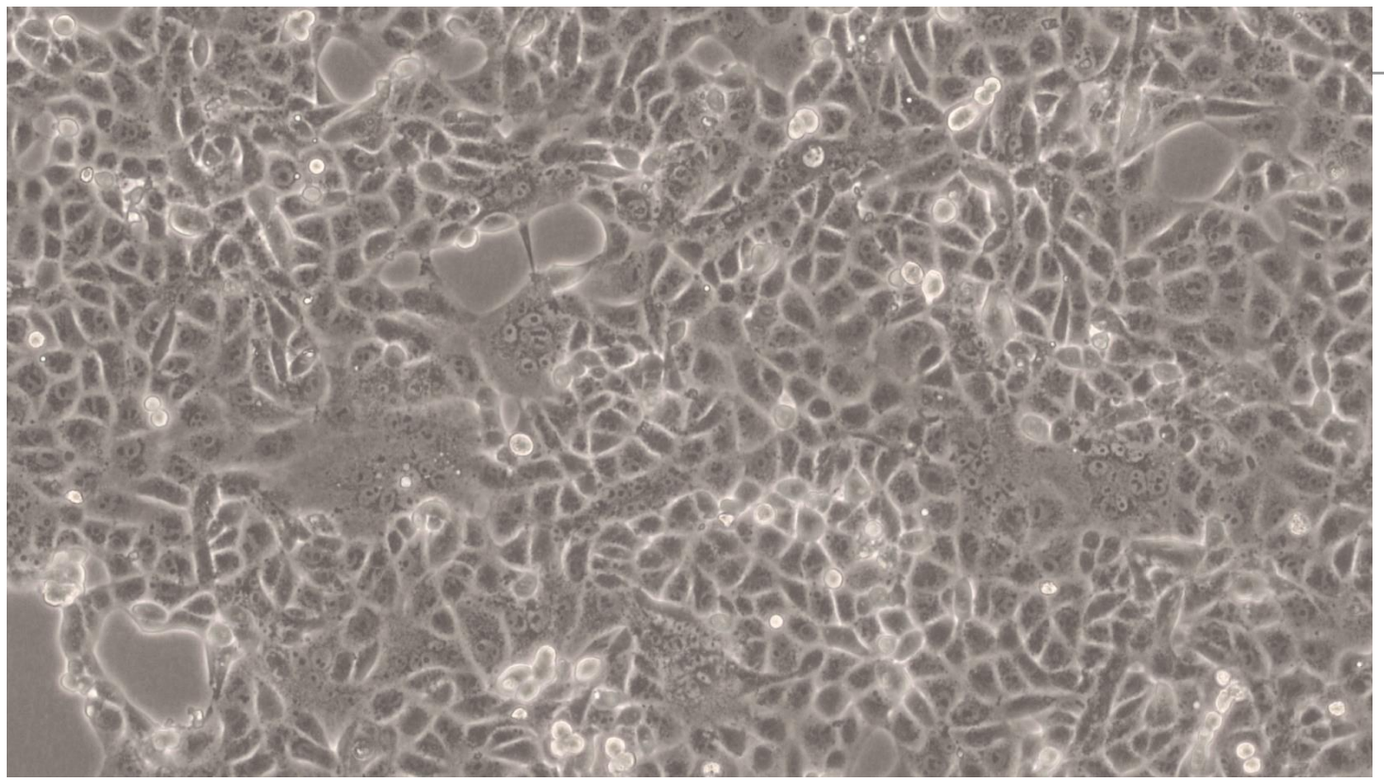
*小鼠,**猪

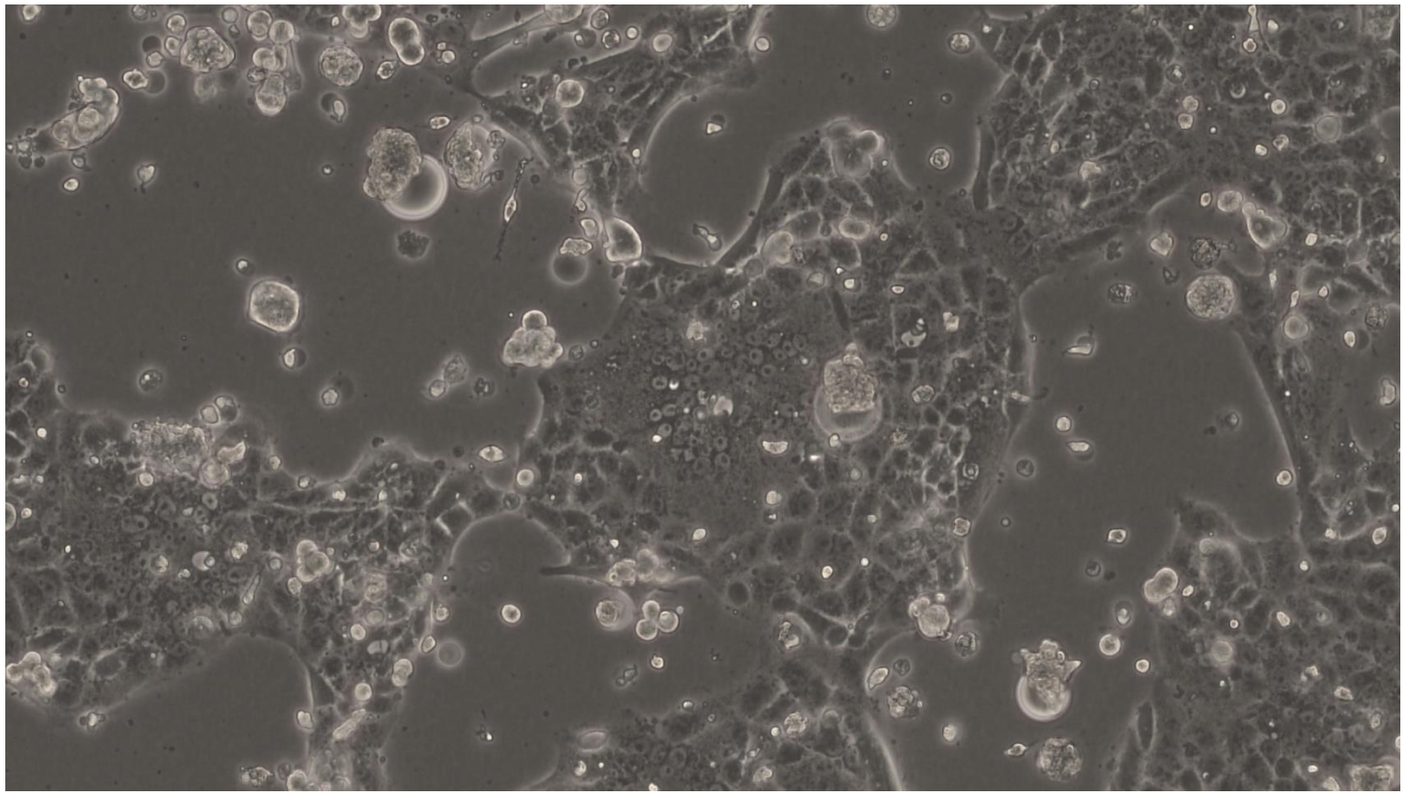
Avian infectious bronchitis replication in vero-Fusion cell



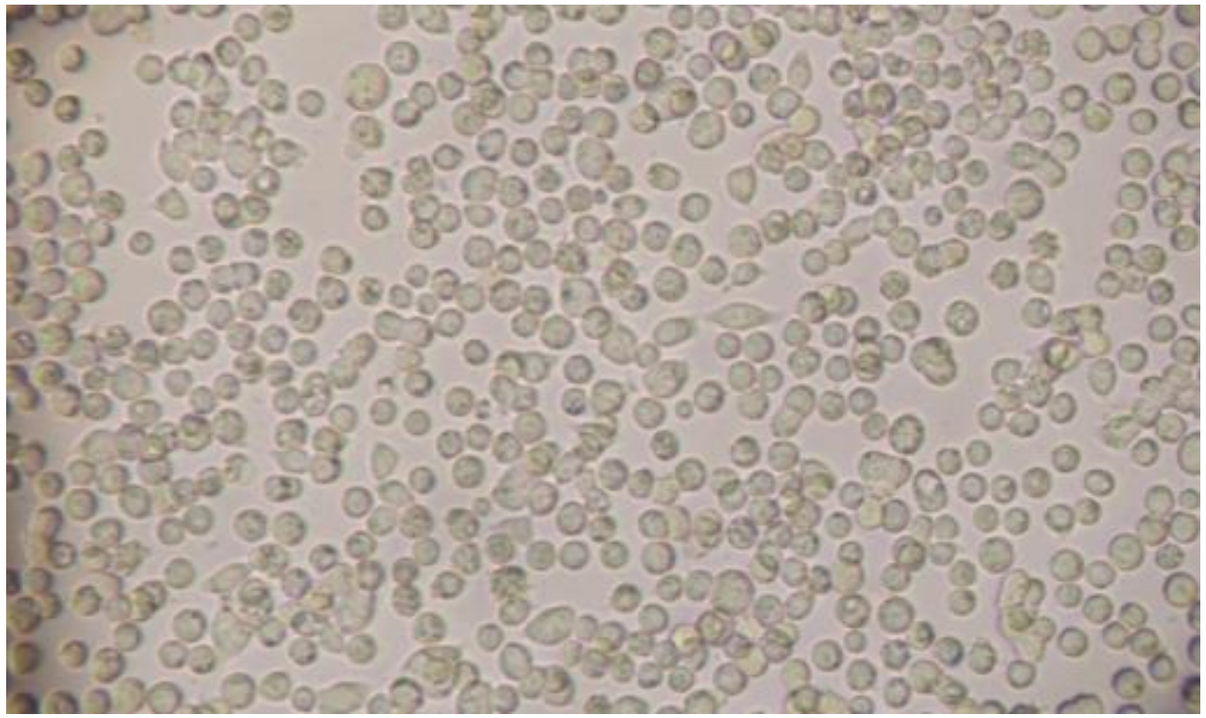


IBV-28/86 replication in vero-Fusion cell

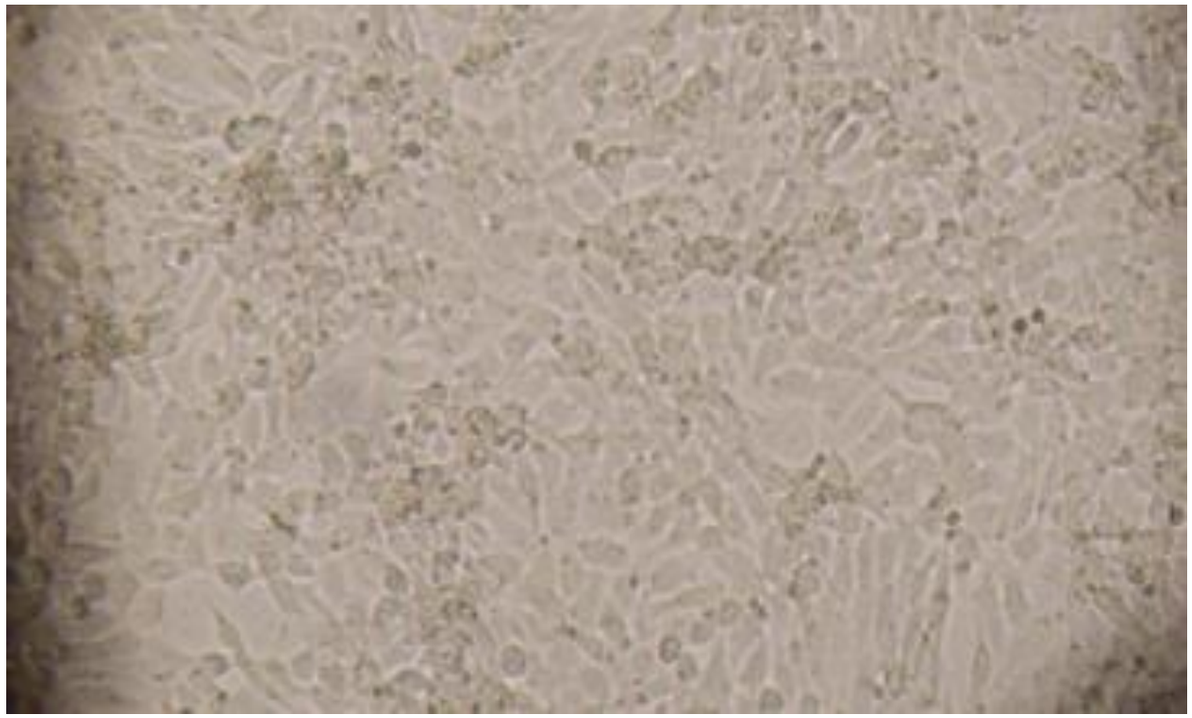




Canine Parvovirus replication in cell lines

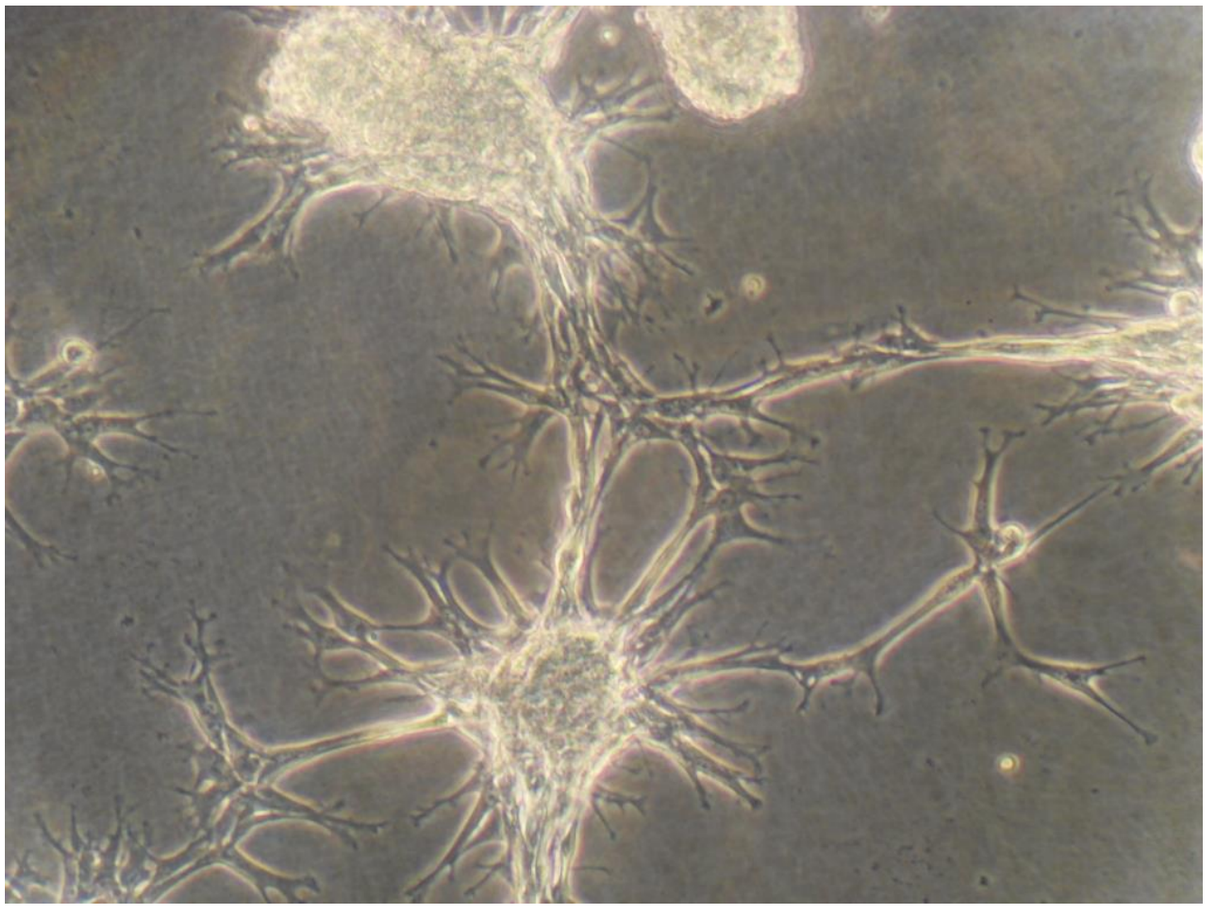


Canine parvocirus/ A549-C3

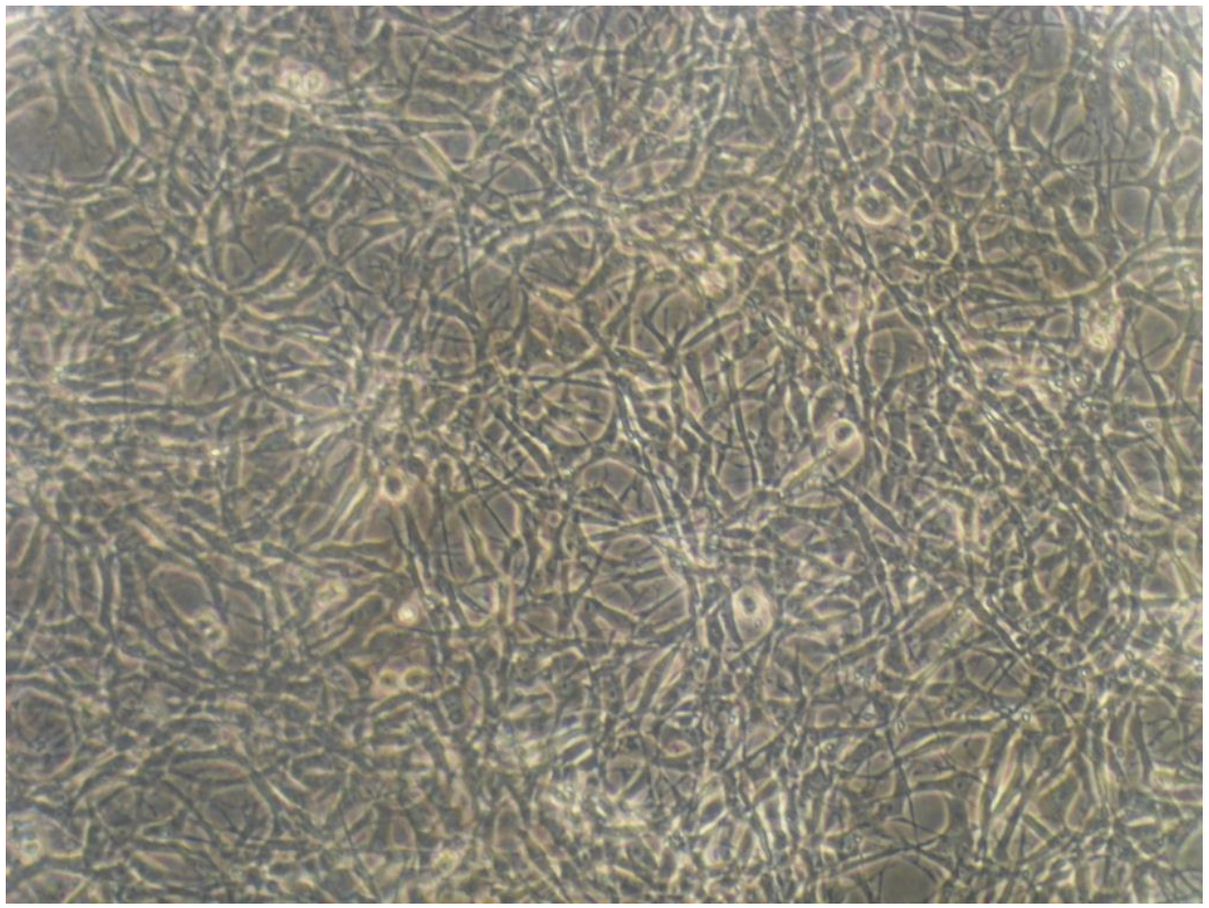


A549-C3_6d_C

ILTV replication in cell lines



BHK21/ILTV



BHK21 Mock inf.

ILT疫苗種毒力致弱研究大綱

ILT種毒

↓

ILT種毒細胞馴化

↓

ILT種毒選殖

↓

ILT種毒雞胚馴化 → ILT抗血清製備

↓ ↘

ILT種毒毒力試驗交叉保護力試驗

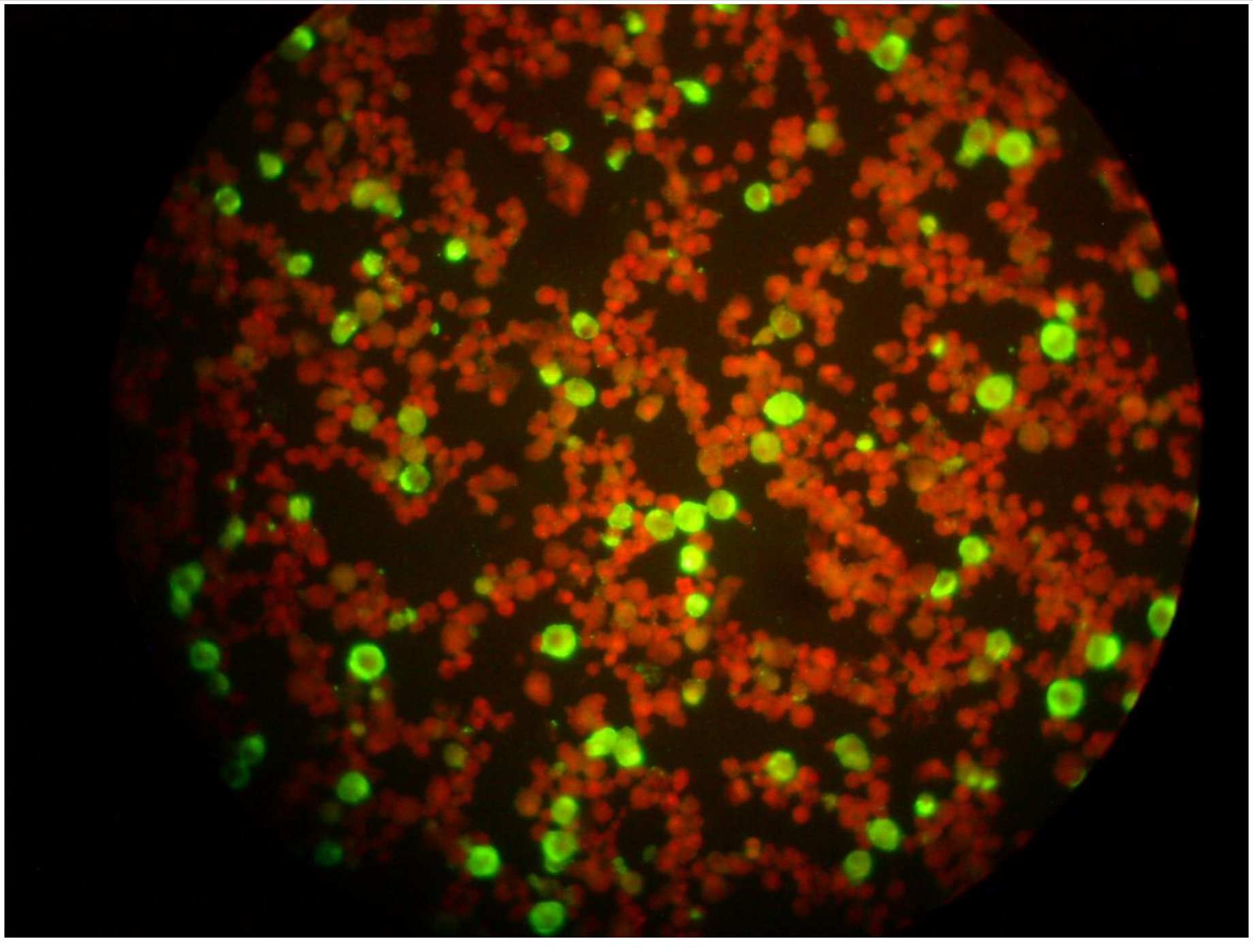
↓

ILT種毒保存

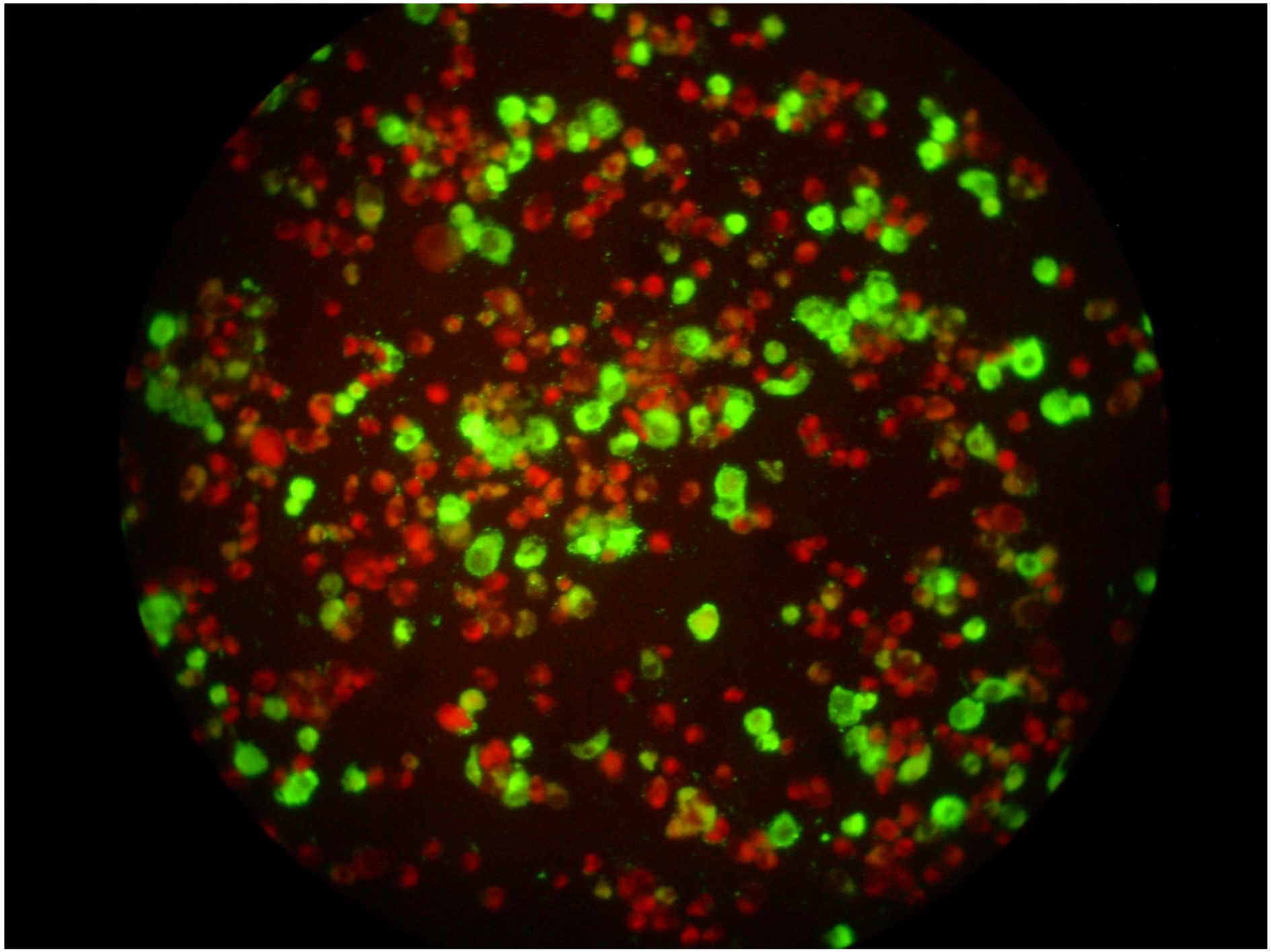
病毒力價消長情形

	P0	P1	P2	P3	P4	P4E1	P4E2
Cells	LMH	LMH	LMH	LMH	LMH	Egg	Egg
ILT-Ct	16.22	14.07	14.1	13.57	13.46		
SPF雞隻接種	+++				-	-	-

IBDV於細胞生長情形



vvIBDV於細胞生長情形



IBDV抗原増殖


TCID50/ml
9
8
7
6
5
4
3
2
1
0
P0
P5
P10
P15
P20
P25
V504-4
V504-5
V504-9
V504-17
V504-21

Table 5 The bursa-body weight ratio (BBR) of the virulence regression test in 1-day-old SPF chickens immunized with IBD-V512 (P20).

Name	Titer[a]/dose $TCID_{50}$	BGL	BHL	BBR at day post vaccination[b] 7D	14D	21D	28D	35D
IBD-V512 (P20)	$10^{5.5}$	0	0	2.24	3.72	4.59	4.16	5.36
Control	-	0	0	2.6	3.18	4.96	4.7	4.2

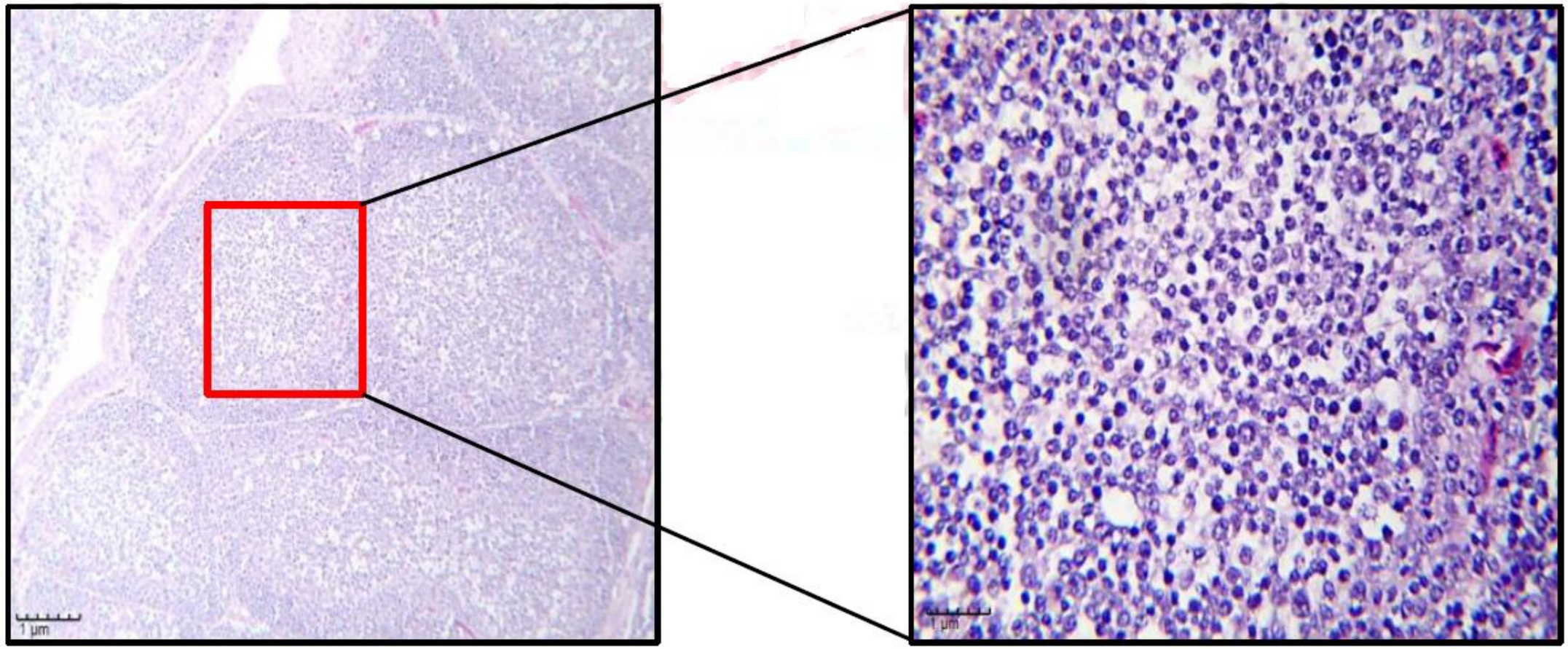
[a] : Titer of the IBD-V512 per dose shown in antilog 10 $TCID_{50}$

[b]: IBD-V512 was bursa/body*1000/5；Control was bursa/body*1000

Table 3 The viral neutralization antibody of IBDV-V512 (P20) was from 5 different $TCID_{50}$ to immune newborn chickens

Titer[a]/dose	**1w**	**2w**	**3w**	**4w**	**5w**	**6w**
$TCID_{50}$	**VN**	**VN**	**VN**	**VN**	**VN**	**VN**
$10^{2.5}$	< 2	2	4	8	16	32
$10^{3.5}$	2	8	16	32	64	64
$10^{4.5}$	4	32	64	128	128	256
$10^{5.5}$	32	256	1024	1024	1024	1024
$10^{6.5}$	64	512	1024	1024	1024	1024
Control	< 2	< 2	< 2	< 2	< 2	< 2

[a]: Titer of the IBD-V512 per dose shown in antilog 10 $TCID_{50}$


1 µm
1 µm

ANIMAL TESTING RESULT – IBD CELL CULTURE VACCINE

I. SCHEDULE

Potency Test : 12th March - 5th May 2018

Serology Test : 26th March - 19th May 2018

Safety Test : 12th March - 16th April 2018

II. MATERIAL AND METHOD

Material:

1. SPF chicken age 14 days
2. Challenge virus : IBDV Indonesian's local strain, 0.1 ml per chicken of 10^3.5 EID50/ml
3. ELISA kit
4. Vaccine samples:

Group	Strain
N (NPUST)	2512-like
A	very virulent
B	Lukert

Vaccines were administered via drinking water at age 14 days with 0.1 ml per dose

Method:

1. Potency test

Vaccine	Dose	Total no. of chicken	Potency test										Safety test					
			ELISA IBD test (week post vaccination)						SN test (week p.v.)		Challenge test (week p.v.)		IBBWR (week post vaccination)					
			1	2	3	4	5	6	3	6	3	6	1	2	3	4	5	6
N	1 dose	60	10	10	10	10	10	10	10	10	10	10	5	5	5	5	5	5
B	1 dose	60	10	10	10	10	10	10	10	10	10	10	5	5	5	5	5	5
A	1 dose	60	10	10	10	10	10	10	10	10	10	10	5	5	5	5	5	5
Control	-	60	10	10	10	10	10	10	10	10	10	10	5	5	5	5	5	5

2. Safety test

Vaccine	Dose	No. of chicken	Safety test	
			Clinical Symptom	IBBWR
			1-35 days p.v.	35 days p.v.
N	10 doses	10	10	10
B	10 doses	10	10	10
A	10 doses	10	10	10
Kontrol	-	10	10	10

Bursa Score Lession (BSL)

0 No lession, normal bursa fabricius
1 1-25% lession at bursa fabricius
2 50-75% lession at bursa fabricius, *yellowish*
3 > 75% lession at bursa fabricius
4 Atrophy

Vaccine passed the standard if not less than 90% vaccinated chickens alive, did not show clinical symptoms of IBD, and BSL <3

III. RESULT AND DISCUSSION

1. Potency test

Group	ELISA IBD titer (week post vaccination)																		% Protection against challenge		IBBWR (week post vaccination)					
	GMT						% protection						CV													
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 3	Week 6	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
N	4 [a]	3885 [b]	4912 [c]	6911 [c]	6911 [c]	6376 [d]	0	100	100	100	100	100	198	33	44	28	41	26	50	50	0.69	0.28	0.41	0.39	0.26	0.62
B	456 [a]	2078 [b]	2304 [b]	3035 [b]	3678 [b]	2289 [b]	10	70	70	90	100	60	89	53	46	40	22	25	40	50	0.51	0.56	0.63	1.04	0.69	1.15
A	2411 [bc]	3384 [b]	3731 [bc]	4861 [b]	5258 [cd]	4009 [c]	70	80	100	90	90	90	33	48	39	30	41	41	60	60	0.21	0.28	0.29	0.37	0.51	0.60
Control	1 [a]	1 [a]	1 [a]	1 [a]	2 [a]	2 [a]	0	0	0	0	0	0	300	200	97	66	277	131	0	0	1.00	1.00	1.00	1.00	1.00	1.00
Standard	2000						≥ 80						≤ 50						≥ 90		Intermediate: > 0.5; Intermediate plus: > 0.3					

1. Potency test

Group	ELISA IBD titer (week post vaccination)											
	GMT						% protection					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
N	4 [a]	3885 [b]	4912 [c]	6911 [c]	6911 [c]	6376 [d]	0	100	100	100	100	100
B	456 [a]	2078 [b]	2304 [b]	3035 [b]	3678 [b]	2289 [b]	10	70	70	90	100	60
A	2411 [bc]	3384 [b]	3731 [bc]	4861 [b]	5258 [cd]	4009 [c]	70	80	100	90	90	90
Control	1 [a]	1 [a]	1 [a]	1 [a]	2 [a]	2 [a]	0	0	0	0	0	0
Standard	2000						≥ 80					

2. Safety test

1. Potency test

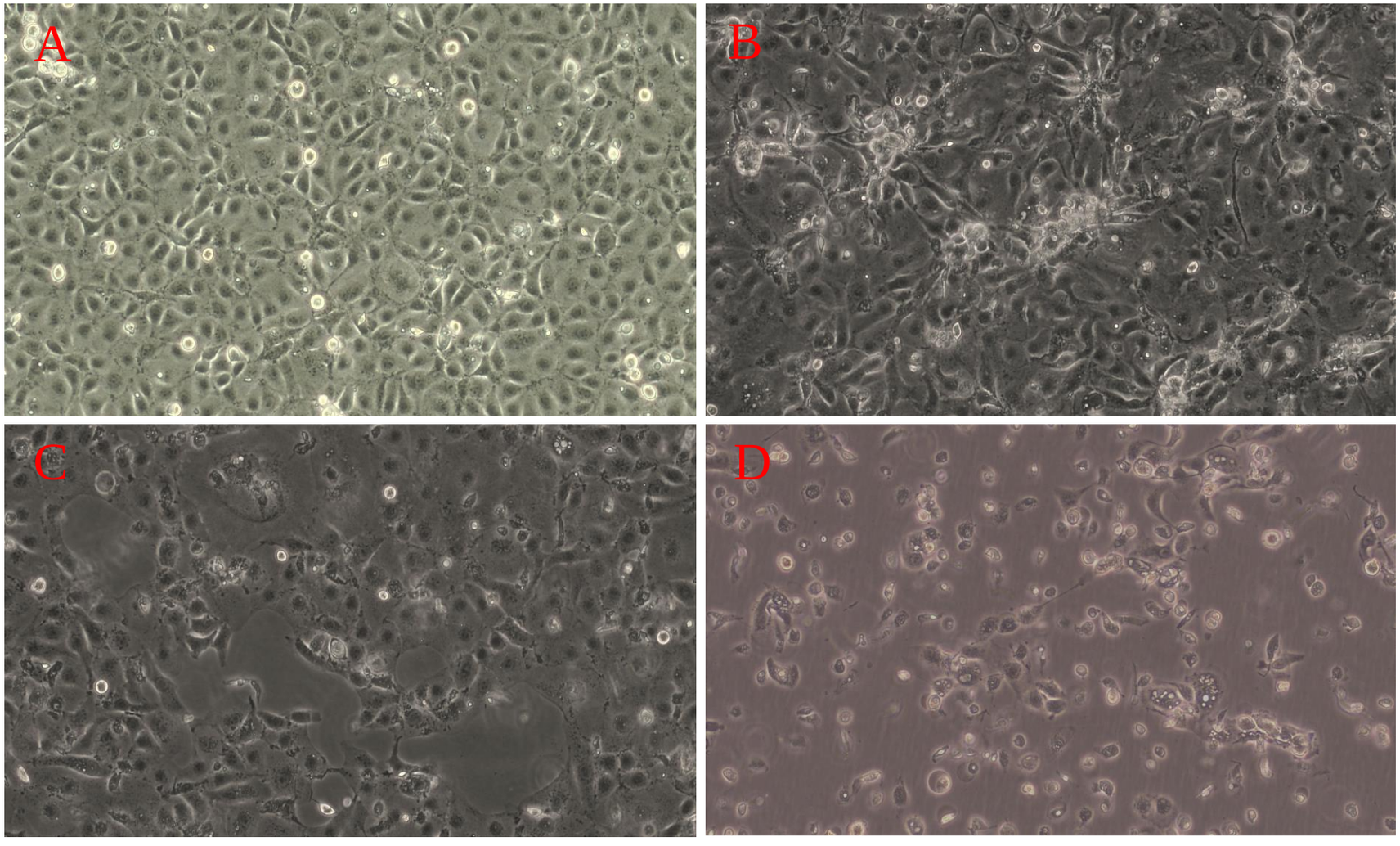
Group	% Protection against challenge		IBBWR (week post vaccination)					
	Week 3	Week 6	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
N	50	50	0.69	0.28	0.41	0.39	0.26	0.62
B	40	50	0.51	0.56	0.63	1.04	0.69	1.15
A	60	60	0.21	0.28	0.29	0.37	0.51	0.60
Control	0	0	1.00	1.00	1.00	1.00	1.00	1.00
Standard	≥ 90		Intermediate: > 0.5; Intermediate plus: > 0.3					

2. Safety test

Observation of clinical symptoms for 35 days post vaccination, IBBWR and BSL for 5 weeks post vaccination

Group	Clinical Symptom	IBBWR	Bursa Score Lession	Conclusion
N	0	0.53	0.8 Bursa : Atrophy (2/10)	Pass
B	0	0.74	0.1	Pass
A	0	0.38	0.8 Bursa : Atrophy (2/10)	Pass
Control	0	1.00	0	
Standard	90% chicken healthy	Intermediate plus : > 0.3 Intemediate : > 0.5	BSL < 3	

Cytopathic effect of Ma-104 cells infected with Rotavirus



Cytopathic effect of Ma-104 cells infected with Rotavirus. A:Control(Only MA-104 cell), B:CPE for 2 dpi, C:CPE for 4 dpi, D:CPE for 6 dpi. * dpi: day post inoculation

次單位疫苗

種毒篩選

C1 C2 C3 C4 C5 .....

蛋白質產量分析

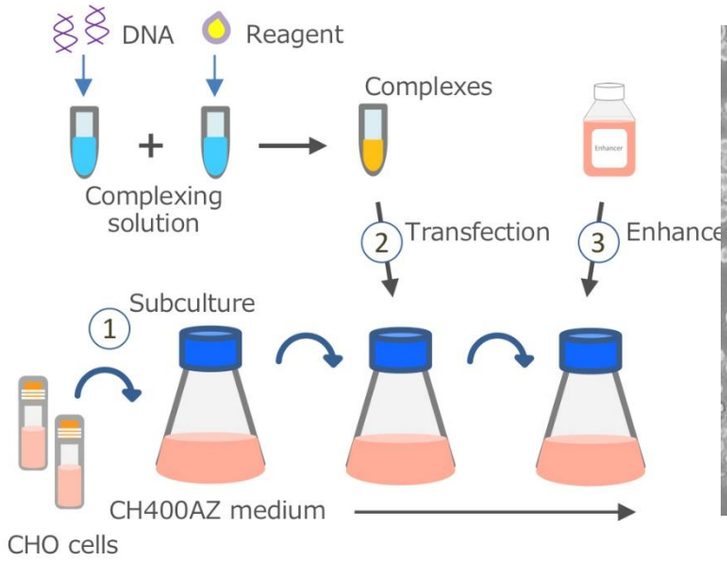
CX → 種毒庫建立

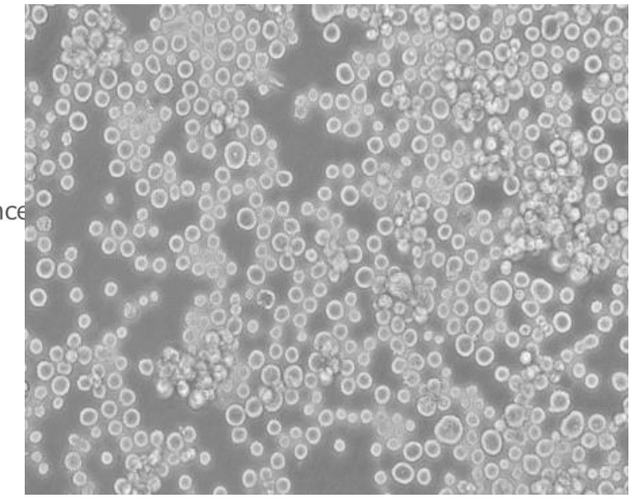
H1 H2 H3 H4 H5 .....

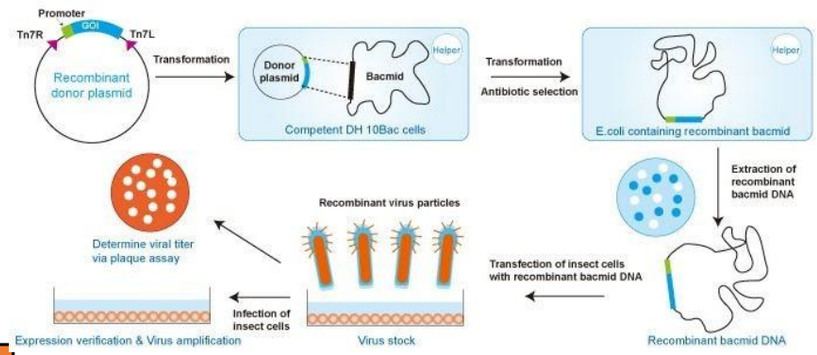
HX → 種細胞庫建立

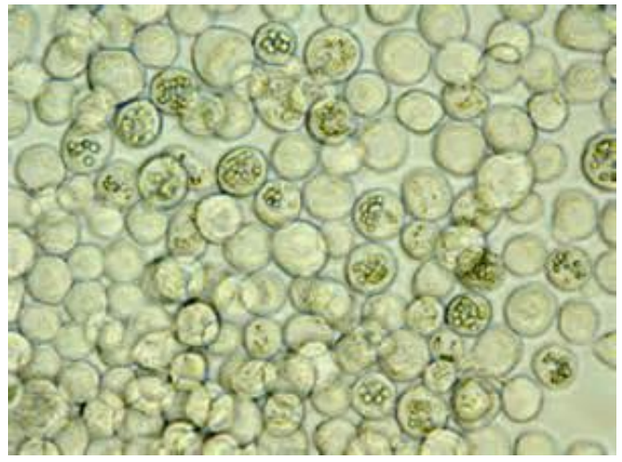
1.穩定性試驗
2.種毒庫品管試驗
3.員工教育訓練

Protocol



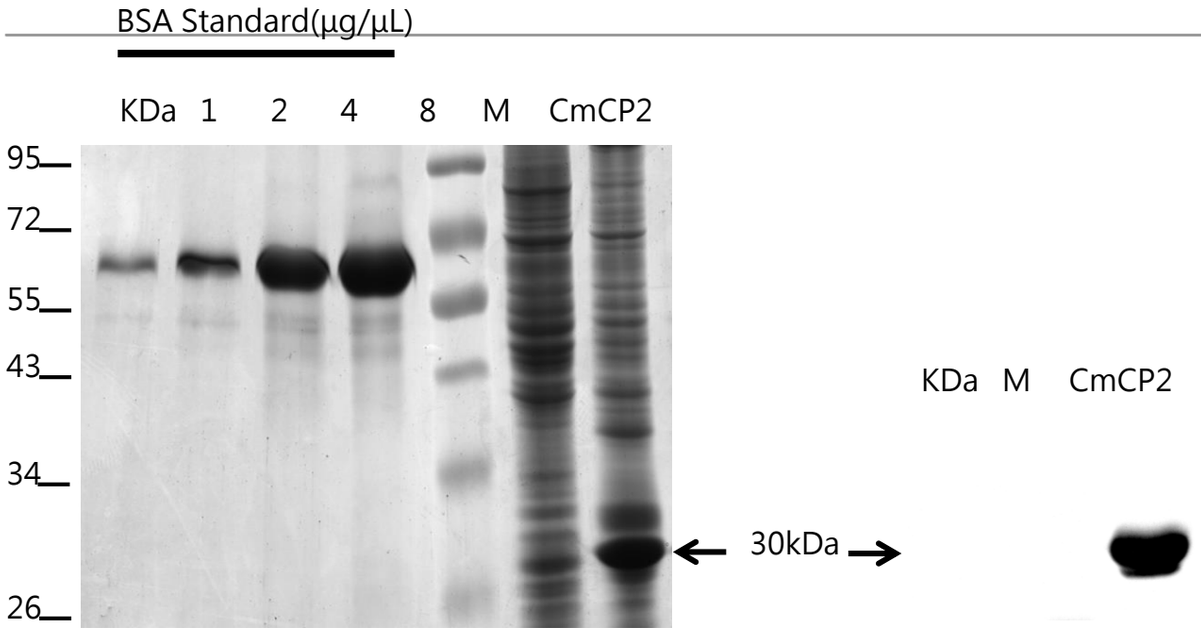




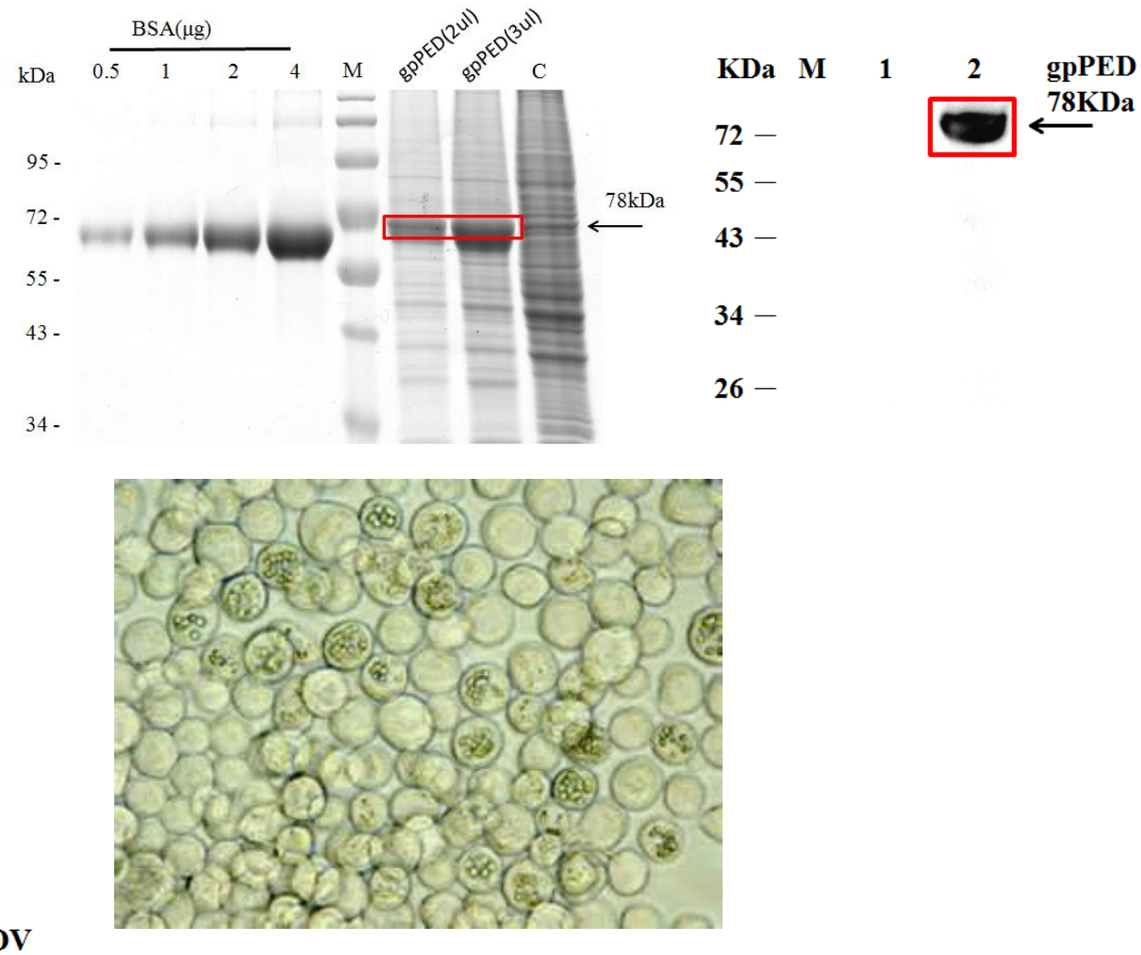


次單位疫苗(桿狀病毒)

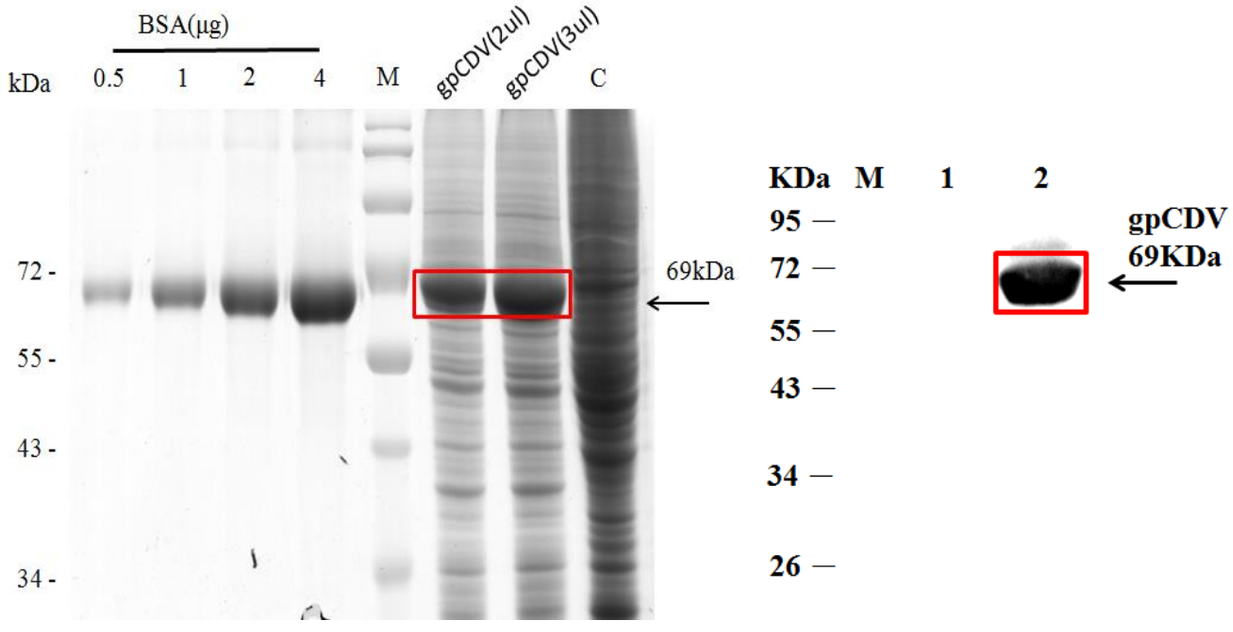
豬第二型環狀病毒(PCV2)抗原蛋白CP2



豬流行性下痢PEDV抗原蛋白S



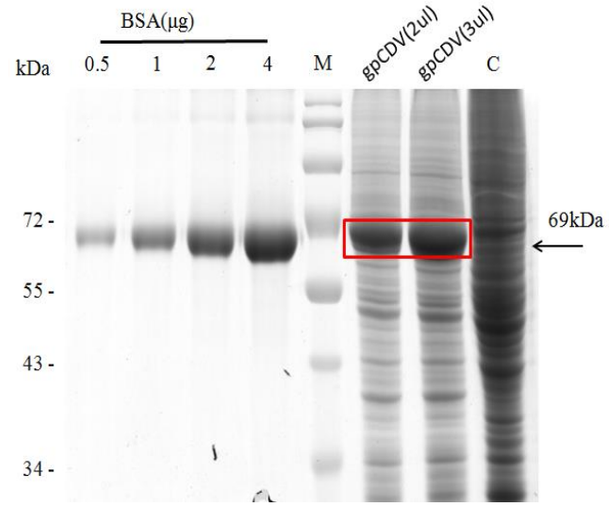
犬瘟熱CDV抗原蛋白H

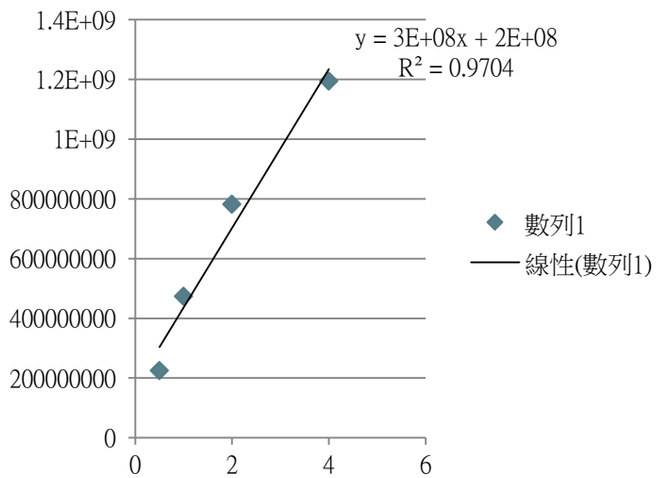


- 蛋白產量100-800 mg/L(搖瓶)200-1000mg/L(發酵槽)
- SDS page即可見表現蛋白

面臨問題

- 產量低：原始蛋白產量???mg/L ➡ ???? g/L
- 工作毒株不穩定
- 種毒庫未建立
- 種毒品管未建立





最佳細胞株篩選

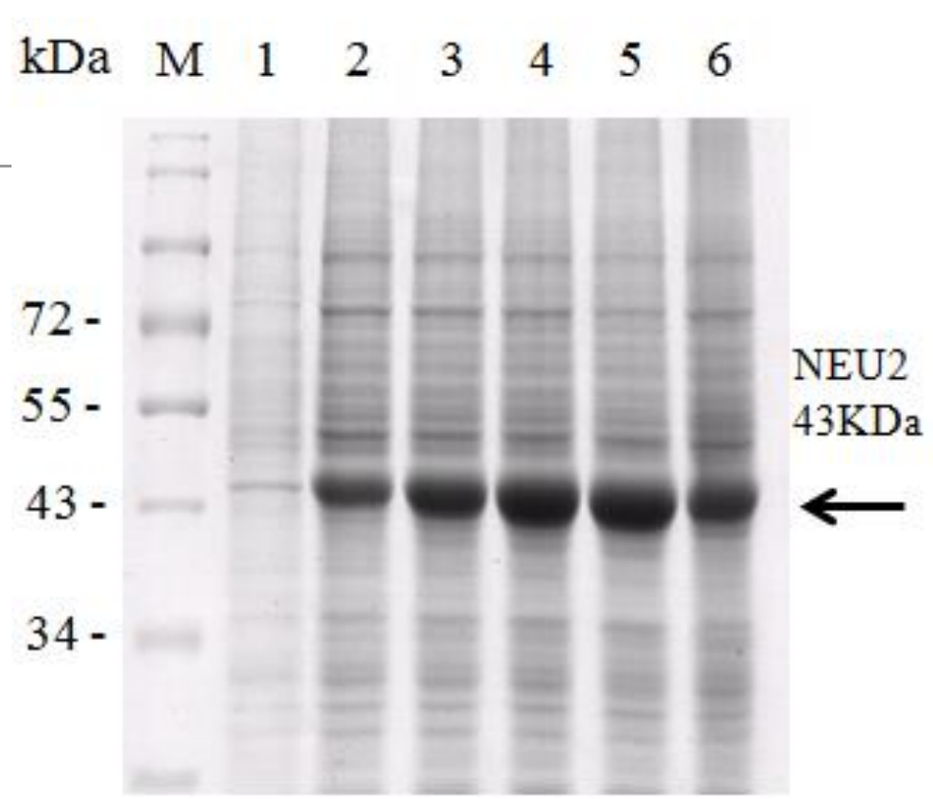
	SF9 C1	SF9 C2	SF9 C3	SF9 C4	SF9 C5	SF9 C6	SF9 C7	SF9 C8	SF9 C9	SF9 C10	SF9 C11	SF9 C12	SF9 C13	SF9 C14	SF9 C15	SF9 C16	SF9 C17	SF9 C18	SF9 C19	SF9 C20
產量 mg/L	117	48	131	210	249	165	N	N	88	135	66	230	188	145	167	220	213	55	131	207

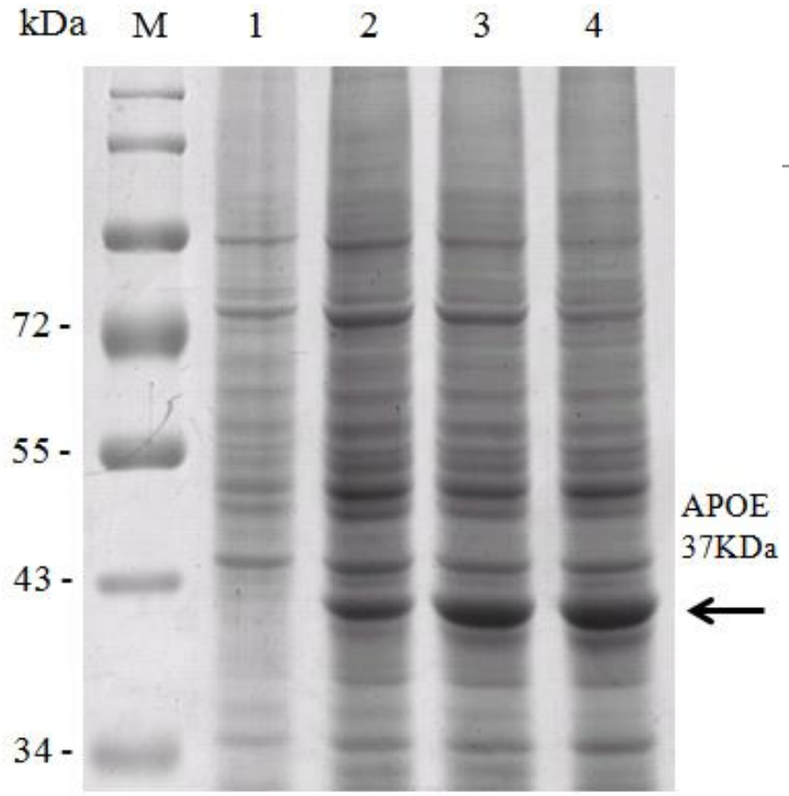
	SF9 C5P1	SF9 C5P2	SF9 C5P3	SF9 C5P4	SF9 C5P5
產量 mg/L	249	256	240	252	244

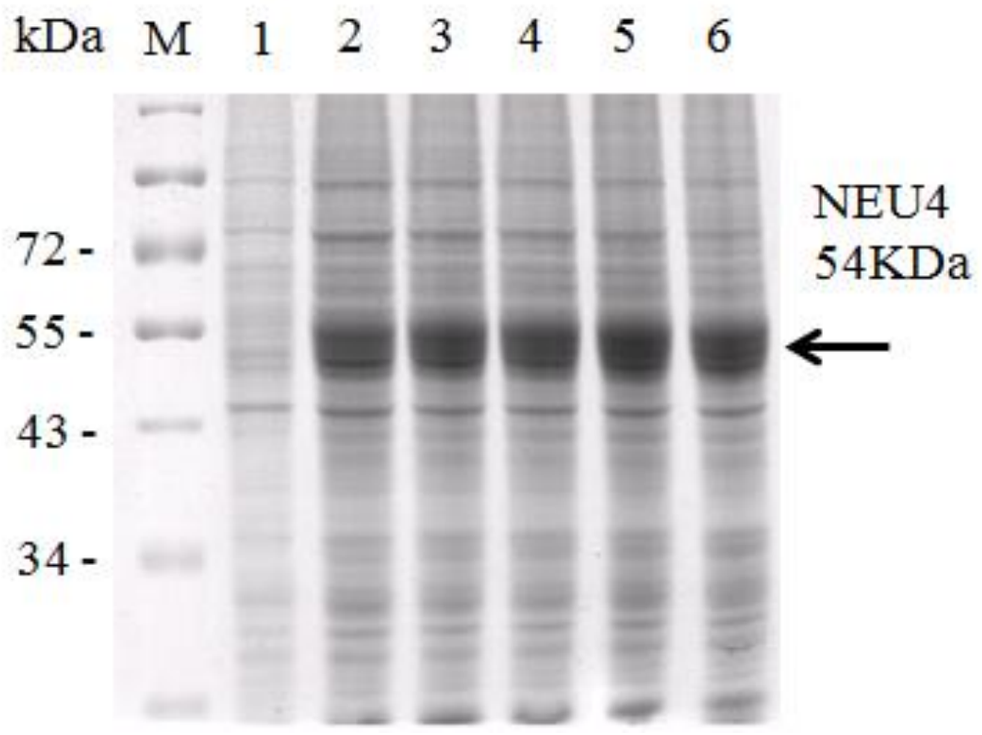
	Hi5 C1	Hi5 C2	Hi5 C3	Hi5 C4	Hi5 C5	Hi5 C6	Hi5 C7	Hi5 C8	Hi5 C9	Hi5 C10
產量 mg/L	221	268	203	244	213	205	188	198	179	232

	Hi5 C2P1	Hi5 C2P2	Hi5 C2P3	Hi5 C2P4	Hi5 C2P5
產量 mg/L	268	232	250	242	248

次單位蛋白(哺乳動物細胞表現系統)







- 蛋白產量60-800 mg/L(搖瓶)100-1000mg/L(發酵槽)
- SDS page即可見表現蛋白

Cell lines created for virus production (I)

Cells	Function	Virus Titer(TCID50/ml)
Vero/207-1 Vero/207-2 Vero/207-3 Vero/207-4 Vero/207-5 Vero/Fusion Vero/Lysis	培養雞傳染性胃腸炎(Inflammatory bowel disease)、傳染性支氣管炎(Infectious bronchitis)、豬流行性下痢(Porcine epidemic diarrhea）、日本腦炎(Japanese encephalitis)	1、IBD→10^8 2、IBV→10^7 3、PED→10^8 4、JE→10^7
Vero-CD150 Vero-CDF	犬瘟熱病毒(Canine distemper virus)	CDV→10^7 CDV→10^7
MA-104/207-1	培養豬輪狀病毒(Porcine rotavirus)	PoRV→10^8
CHO-207	Mammalian cell expression	ASAH2
293F-207	Mammalian cell expression	ASAH2
PK-15 PK-15-C1 PK-15-C2 PK-15-C3 PK-15-C4	培養豬假性狂犬病(Pseudo rabies)、豬傳染性胃腸炎(Transmissible Gastroenteritis)、第二型豬環狀病毒（Porcine circovirus type 2）、豬小病毒. (Porcine parvovirus)、豬瘟病毒(Hog cholera virus)	1、PR→10^9 2、TGE→10^8 3、PCV2→$10^{6.5}$ 4、PPV→10^8 5、HCV→$10^{6.5}$
CEK207	培養傳染性支氣管炎(Infectious bronchitis)	
LMH207	培養禽腺病毒(EDS)、雞傳染性喉頭氣管炎(Infectious Laryngotracheitis)	1、EDS→10^7 2、ILT→10^7
DF-1-207	培養雞新城病(Newcastle disease LaSota)	HA test→2^{10}
MSB-1-207	培養雞傳染性貧血(Chicken infectious anemia)	CAV→10^7

Cell lines created for virus production (II)

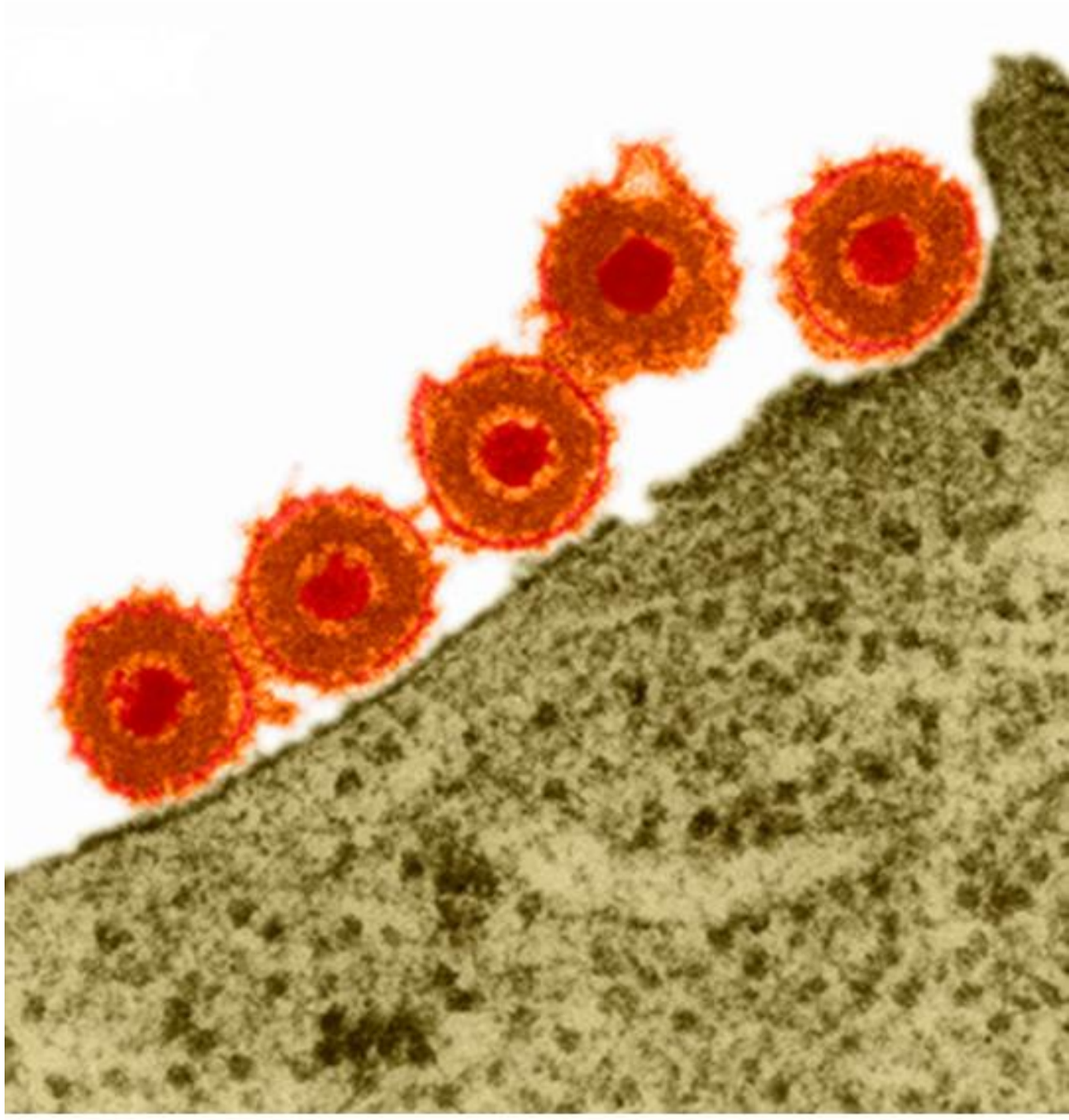
Cells	Function	Virus Titer
MDBK 207	培養牛病毒性下痢(Bovine viral diarrhea virus)	BVDV→10^8
MDCK207	培養禽流感(Avian Influenza)	
CRFK207	培養犬细小病毒第二型(Canine parvovirus type2a；Canine parvovirus type2b；Canine parvovirus type2c)、貓傳染性腹膜炎(Feline Infectious Peritonitis)	1、CPV：HA test→2^8 2、FIP→10^8
F81-207	培養犬细小病毒第二型(Canine parvovirus type2a；Canine parvovirus type2b；Canine parvovirus type2c)、貓瘟病毒(Feline parvovirus)、貓疱疹病毒(Feline herpesvirus)	1、CPV：HA test→10^8 2、FPV:檢體難取得 3、FHV→10^7
BHK21-Fusion **BHK21-Lysis** **BHK21-207**	培養雞新城病(Newcastle disease)、家禽里奧病毒(Avian reovirus、牛流行熱病毒(Bovine ephemeral fever virus)、狂犬病病毒(Rabies virus)、口蹄疫（Foot and Mouth Disease）	1、HA test→2^8 2、ARV→10^7 3、BEFV→10^8

病毒培養參考力價

Vaccine	Virus strain	Antigen production	Virus titer(TCID50)
Pseudorabies live vaccine	BathaK-61	Cell culture	10^9
PRRS inactived vaccine	N	Cell culture	10^7
PRRS inactived vaccine	S	Cell culture	10^7
PRRS inactived vaccine	E	Cell culture	10^7.5
PRRS inactived vaccine	W	Cell culture	10^7
PRRS inactived vaccine	classical	Cell culture	10^7
Porcine epidemic diarrhea live vaccine	CV777	Cell culture	10^10
Porcine epidemic diarrhea inactivated vaccine	Wild type KC/14	Cell culture	10^8
Porcine epidemic diarrhea inactivated vaccine	Wild type CL/14	Cell culture	10^8
Porcine circovirus type 2 inactivated vaccine	Wild type DV/12(2b)	Cell culture	10^7.5
Porcine circovirus type 2 inactivated vaccine	Wild type DV/12(2d)	Cell culture	10^7.5
Porcine Transmissible gastroenteritis live vaccine		Cell culture	10^7
Avian reovirus live vaccine(malabsorption syndrome)	OS161	Cell culture	10^8.5
Avian reovirus live vaccine (viral arthritis syndrome)	S1133	Cell culture	10^9
Avian reovirus live vaccine (viral arthritis syndrome)	750505	Cell culture	10^9
Avian infectious bronchitis live vaccine	H120	Cell culture	10^7
Avian infectious bronchitis live vaccine	Ma-5	Cell culture	10^6
Avian infectious bronchitis live vaccine	M41	Cell culture	10^6
Avian infectious bronchitis(nephritis) live vaccine	TW-1	Cell culture	10^7
Avian infectious bronchitis(nephritis) live vaccine	TW-2	Cell culture	10^8
Avian infectious bronchitis(glandular stomach type) live vaccine	Wild type	Cell culture	10^8
Infectious Bursa Disease live vaccine	luker	Cell culture	10^8.5
Infectious Bursa Disease live vaccine	MB43	Cell culture	10^8
Infectious Bursa Disease live vaccine	2512	Cell culture	10^8.5
Infectious Bursa Disease live vaccine	medivac	Cell culture	10^8
Infectious Bursa Disease live vaccine	Wild type vvIBD	Cell culture	10^7
Infectious Bursa Disease live vaccine	Wild type IBD	Cell culture	10^8
Infectious Laryngotracheitis live vaccine		Cell culture	10^7
Newcastle Disease Genotype VII inactivated vaccine	TW-V158-VIId	Cell culture	10^8
Newcastle Disease Genotype VII inactivated vaccine	TW-V301-VIIe	Cell culture	10^8
Newcastle Disease Genotype VII inactivated vaccine	China-V178VIIe	Cell culture	10^8
Avian reovirus inactivated vaccine (viral arthritis syndrome)	750505-like	Cell culture	10^9
Avian reovirus inactivated vaccine (viral arthritis syndrome)	S1133	Cell culture	10^9
Avian reovirus inactivated vaccine (viral arthritis syndrome)	1733	Cell culture	10^8
Avian reovirus inactivated vaccine (malabsorption syndrome)	2408	Cell culture	10^8
Avian reovirus inactivated vaccine (malabsorption syndrome)	OS161-like	Cell culture	10^8.5
Fowl adenovirus Serotype IV inactivated vaccine	Wild type	Cell culture	10^8
Canine distemper inactivated vaccine	2014 Wild type	Cell culture	10^7
Canine parvovirus inactivated vaccine	2010 Wild type	Cell culture	2^10 HAU
Feline infectious peritonitis inactivated vaccine		Cell culture	10^9
Feline Herpesvirus inactivated vaccine	2016 Wild type	Cell culture	10^7
Koi herpesvirus inactivated vaccine	2013 Wild type	Cell culture	10^8.5
Bovine ephemeral fever inactivated vaccine	2014 Wild type	Cell culture	10^8
Bovine viral diarrhea inactivated vaccine	2019 Wild type	Cell culture	10^8
Bovine enterovirus inactivated vaccine	2019 Wild type	Cell culture	10^8

2

Expression of virus receptors in cells



Principles

- Receptors on cell surface are the keys for the virus to enter the cell.
- Virus cannot enter cells without the receptors.
- Thus, expression of the necessary receptors may make cells susceptible to virus infection.

Fcγ Receptor IIA gene (956bp)

- **Fc receptor (FcR)**

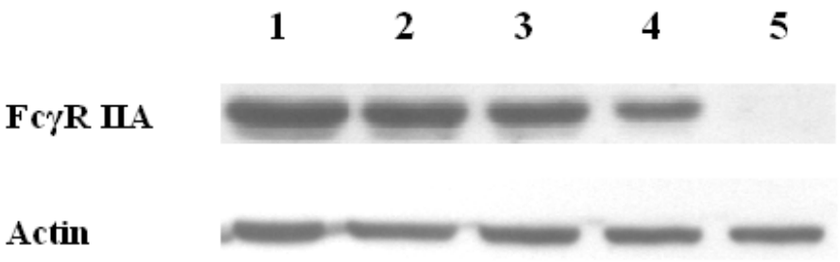
- **a multigene family of integral membrane glycoproteins**
- **mediated entry of infectious dengue virus immune complexes into monocytes/macrophages**
- **is hypothesized to be a key event in the pathogenesis of complicated dengue fever**
- **FcRIA (CD64, 73kDa) and FcRIIA (CD32, 40kDa)**

FcrRIIA/poDNA3.1(-) recombinant plasmid sequencing

Sequences producing significant alignments:
(Click headers to sort columns)

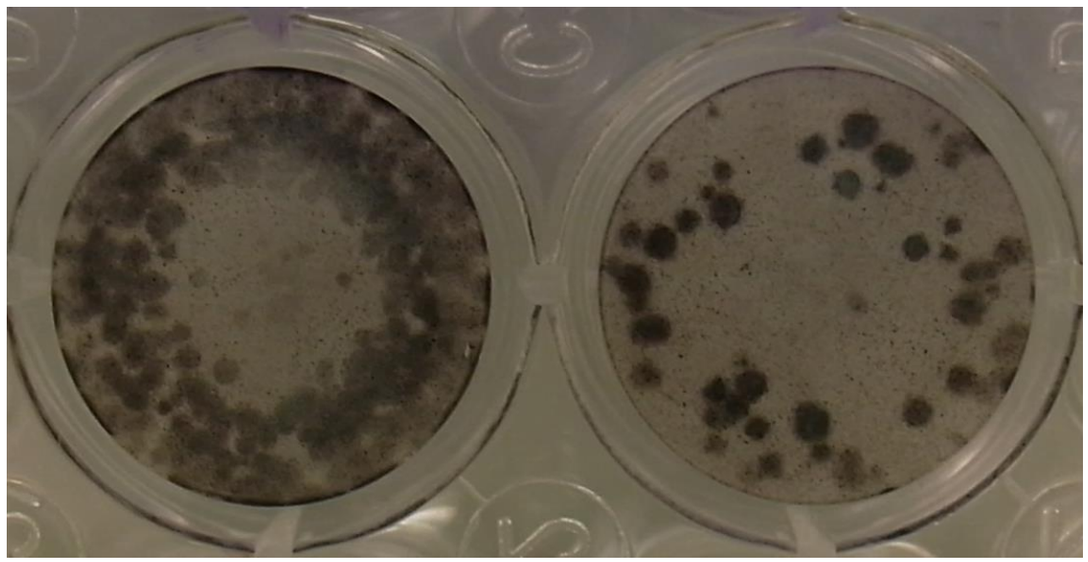
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NM_001136219.1	Homo sapiens Fc fragment of IgG, low affinity IIa, receptor (CD32)	1079	1413	69%	0.0	99%
AK315225.1	Homo sapiens cDNA, FLJ96223, highly similar to Homo sapiens Fc fr	1079	1413	69%	0.0	99%
AK225438.1	Homo sapiens mRNA for Low affinity immunoglobulin gamma Fc reg	1079	1413	69%	0.0	99%
Y00644.1	Human FcRII mRNA for immunoglobulin G receptor	1074	1407	69%	0.0	99%
X62572.1	H.sapiens RNA for Fc receptor, PC23	1074	1407	69%	0.0	99%
M28697.1	Human low-affinity IgG Fc receptor (alpha-Fc-gamma-RII) mRNA, c	1074	1407	69%	0.0	99%
J03619.1	Human immunoglobulin G Fc receptor mRNA, complete cds	1074	1407	69%	0.0	99%
M31932.1	Human IgG low affinity Fc fragment receptor (FcRIIa) mRNA, compl	1074	1407	69%	0.0	99%
NM_021642.3	Homo sapiens Fc fragment of IgG, low affinity IIa, receptor (CD32)	1061	1394	69%	0.0	98%
CU676732.1	Synthetic construct Homo sapiens gateway clone IMAGE:100022215	1061	1061	51%	0.0	98%
DQ895228.2	Synthetic construct Homo sapiens clone IMAGE:100009688; FLH182	1061	1392	69%	0.0	98%
DQ892037.2	Synthetic construct clone IMAGE:100004667; FLH182559.01X; RZPD	1061	1392	69%	0.0	98%
BC020823.1	Homo sapiens Fc fragment of IgG, low affinity IIa, receptor (CD32),	1061	1394	69%	0.0	98%
CR593871.1	full-length cDNA clone CS0DI084YH01 of Placenta Cot 25-normalize	1059	1392	68%	0.0	99%

BHK-21 cells expressing the FcγR IIA receptor allows higher Dengue virus replication



Lane1-4:BHK-21 with Fcγ IIA receptor
Lane 5: BHK-21

Plaque formation



BHK-21 with FcgR IIA BHK-21

-Positive control

(positive serum + virus)

-Viral back titration

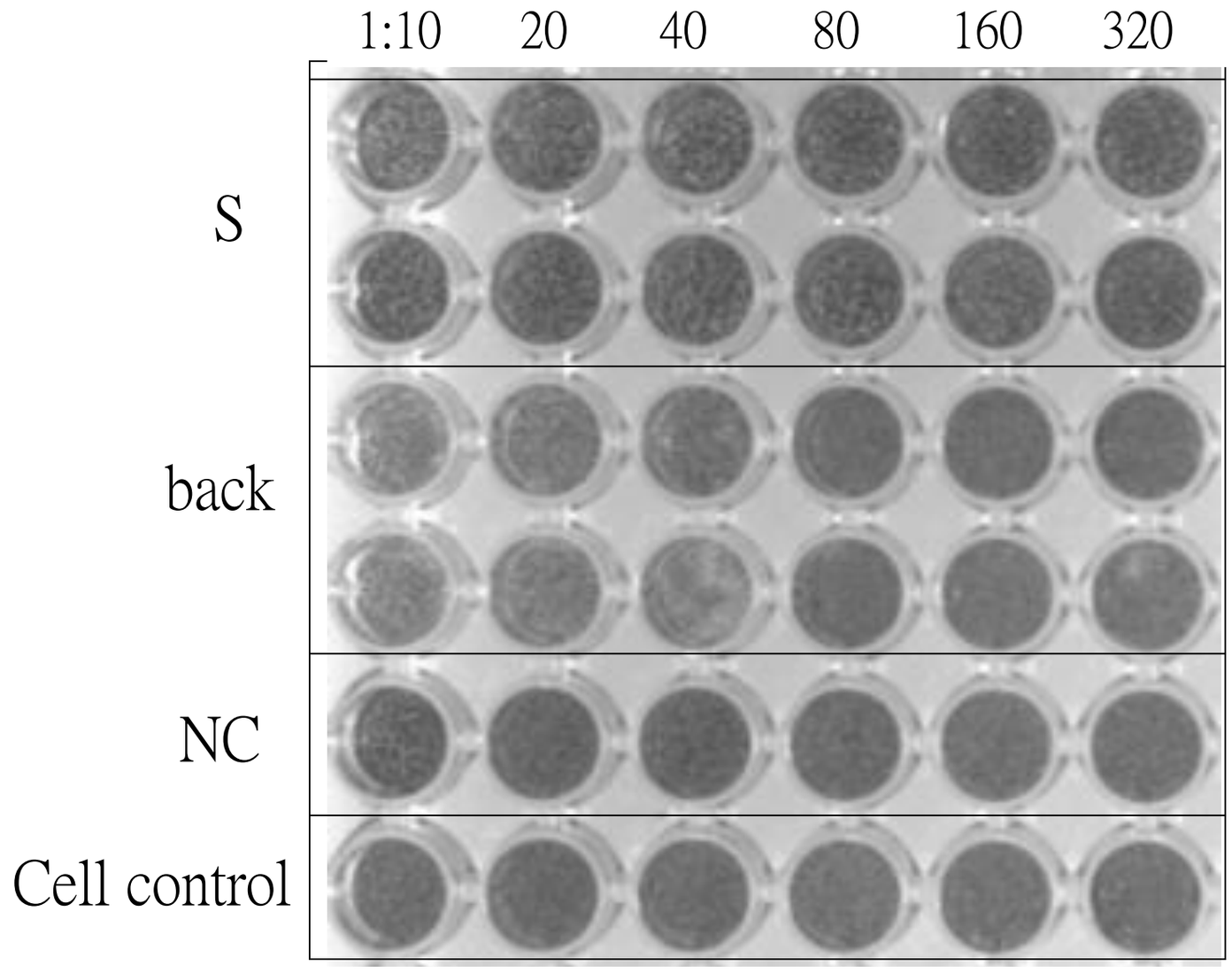
(virus 10X serial dilution,)

-Negative control

(Normal serum, no virus)

-Cell control

(no serum and virus)



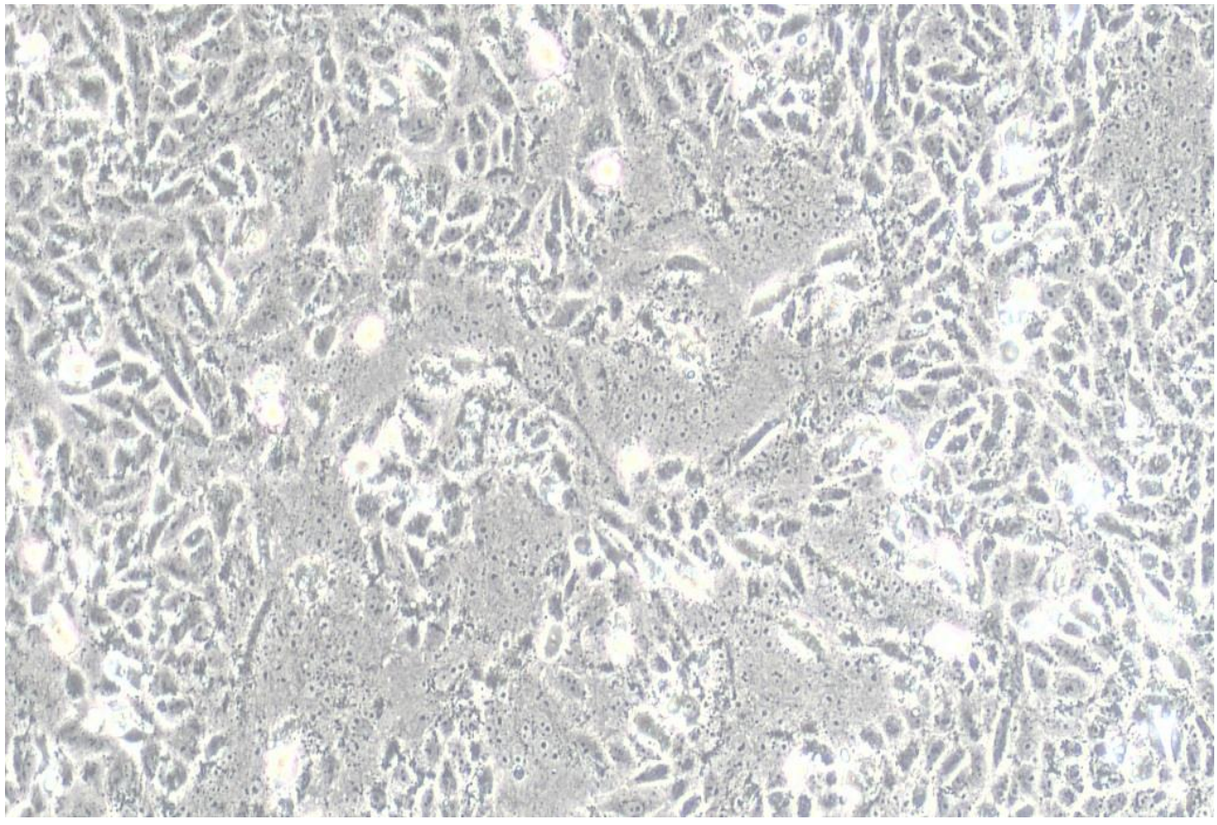
Sample No. 91076 (DEN2)

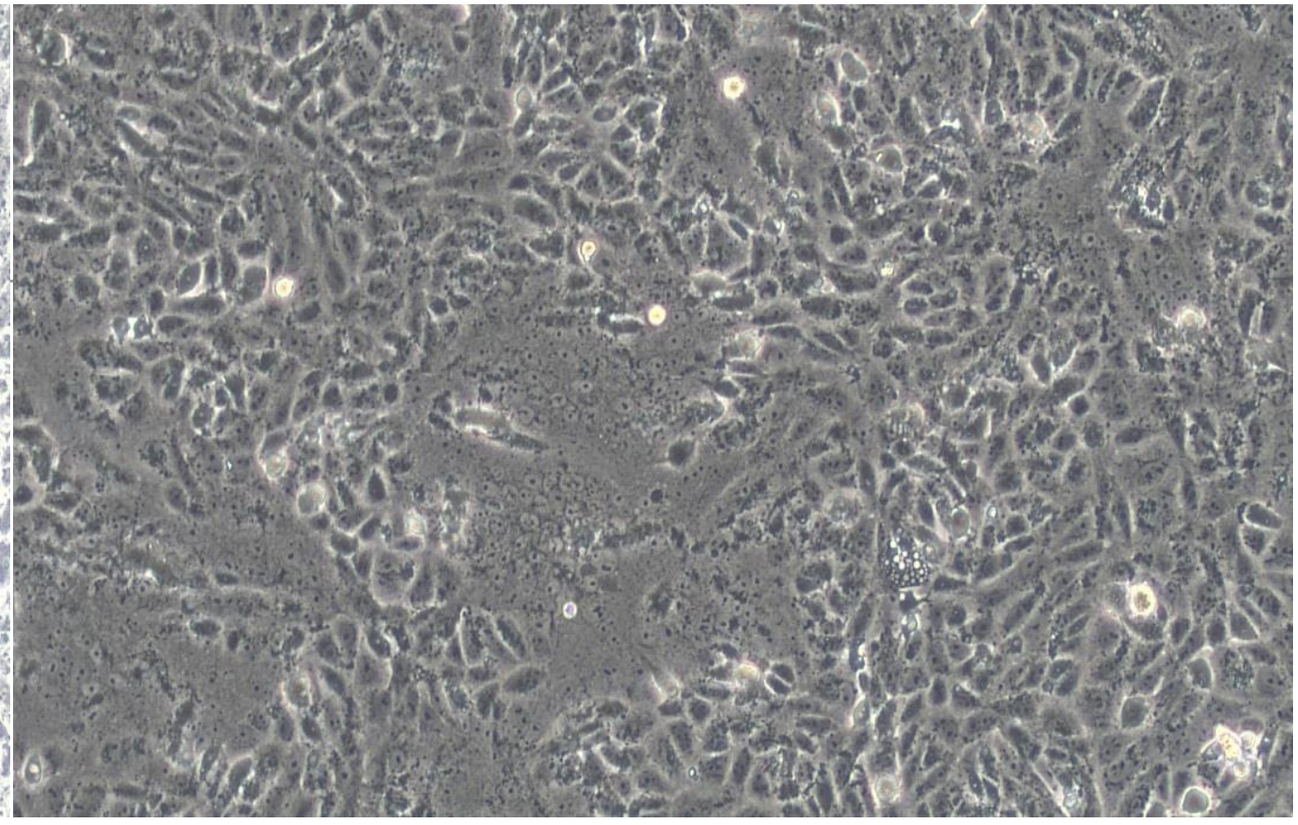
Titer >1:320

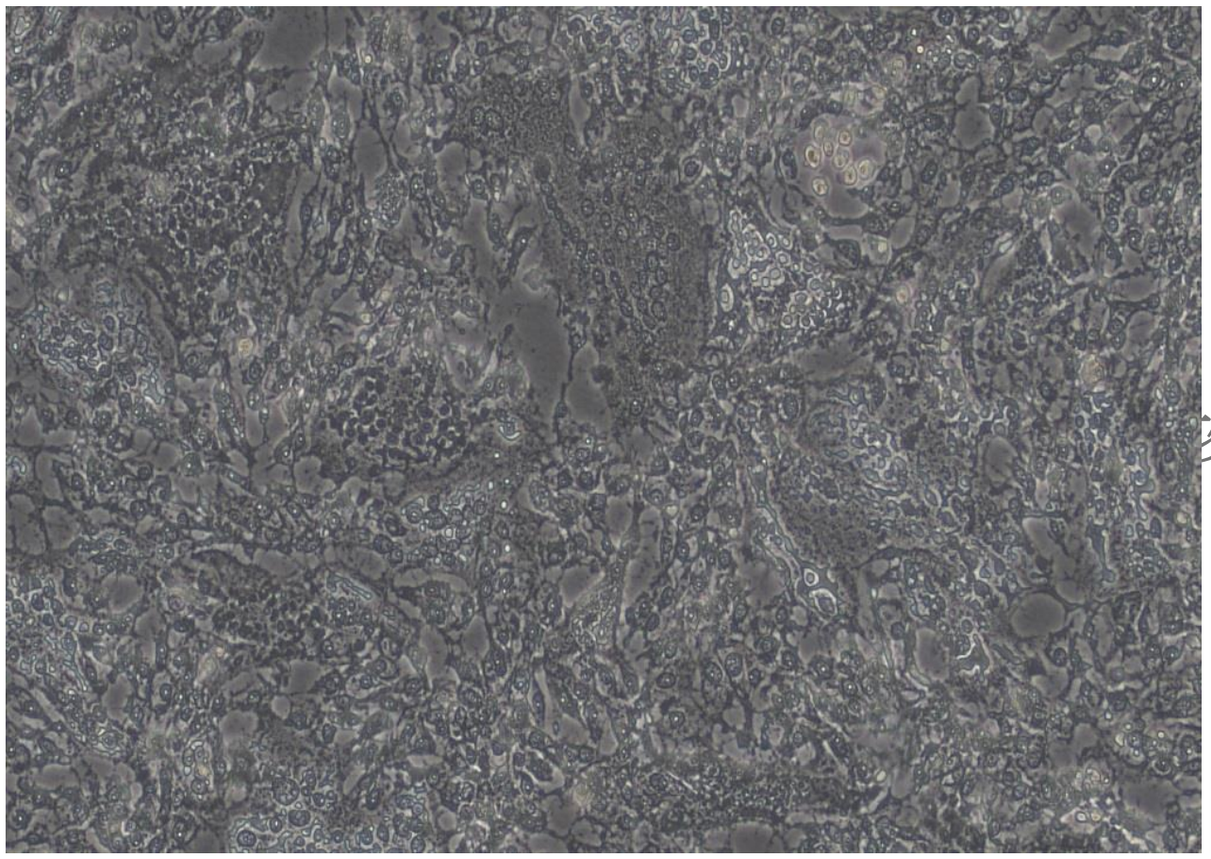
- FcR II: Dengue virus

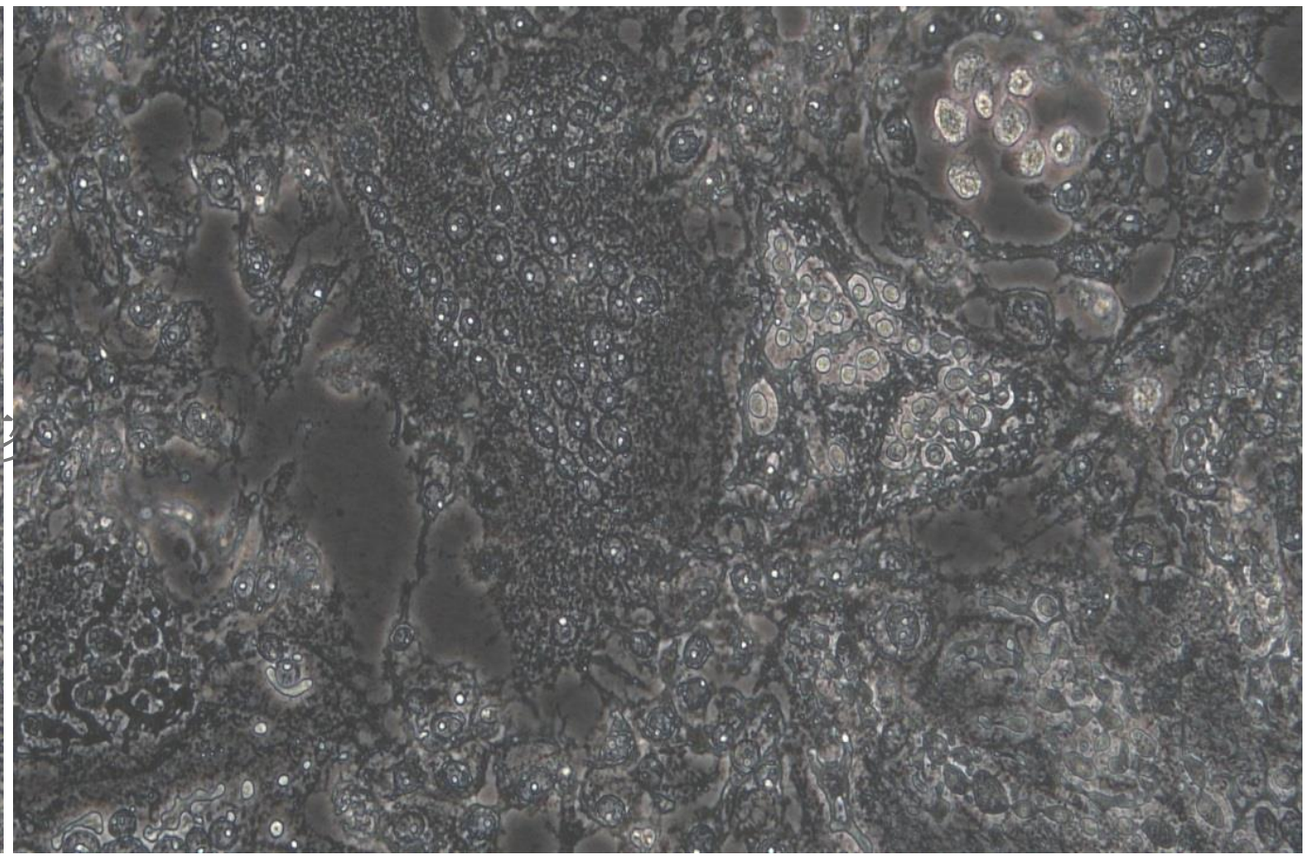
- CD46: Canine distemper virus

CDV Vero 攻毒後第一天









細胞之選擇

分組	Vero	Vero-CD150	Vero-F
外源蛋白表現	None	犬隻CD150	犬隻CDV-F
轉染載體	None	pDisplay	pcDNA 3.1
細胞代數	P40	P9	P8

分組	**Vero**		**Vero-CD150**	**Vero-F**
成份	MEM	MEM TPCK-TV	MEM TPCK-TV	MEM TPCK-TV
Ct-1	14.26		ND	ND
Ct-2	ND	13.36	17.7	ND
Ct-3	21.19	15.64	25.06	11.02
Note				***

攻毒/培養條件

分组	A	B	C	E
成份	MEM TPCK-TV	MEM TPCK-TV	MEM TPCK-TV	MEM TPCK-TV
Cells	Vero-F	Vero-F	Vero-F	Vero-F
病毒稀释倍数	32	32	100	3
天数	3	3	2	2
细胞情形	CPE 90%	Death 70%	CPE 90%	Death 70%
TCID50(log10)/mL	6.9	7.4	7.4	5.7
Note		**	**	

病毒馴化消長情形

	P0	**P1**	**P2**	**P3**	**P4**
Cells	Vero	Vero	Vero-F	Vero-F	Vero-F
Ct	15.59	14.26	13.36	11	ND
TCID50 (log10)/ml	6.5	ND	6.9	7.4	7.4
Note				*	*

穩定性試験

	P3	P4	P5	P6	P7	P8	P9
Cells	Vero-F	Vero-F	Vero-F	Vero-F	Vero-F	Vero-F	Vero-F
Ct	11	ND	ND	ND	ND	ND	ND
TCID50 (log10)/ml	7.4	7.4	7.1	7.3	7	7.4	7.4

基因改造動物用生物藥品分類

依管制生物體之特性,分為下列三類:

第一類基改生物藥品死毒、死菌、次單位疫苗、單株抗體、基因重組蛋白質及不具複製能力之載體等產品。

第二類基改生物藥品:將基因剔除之活菌(毒)疫苗產品。

第三類基改生物藥品:帶有外源基因插入載體之活菌(毒)疫苗產品。

基因改造生物藥品所需風險評估文件

基因改造動物用生物藥品廠商自行審核書面報告

審查項目			自行審核	頁次
品質文件			☐	P
	用於生產種株的原始材料		☐	P
	構築載體使用的遺傳物質		☐	P
	載體		☐	P
	插入的核酸序列		☐	P
	基因工程疫苗最終產品的特性		☐	P
安全性及風險評估文件			☐	P
	危害鑑定		☐	P
		動物安全性	☐	P
		對象動物之安全性	☐	P
		非對象動物之安全性	☐	P
		公共衛生安全性	☐	P
		環境安全性	☐	P
	田間安全性試驗前對環境釋放的評估		☐	P
		釋放地點的位置	☐	P
		釋放地點的特性	☐	P
		人員	☐	P
		實驗設計	☐	P
		擴散和散播的潛力	☐	P
		在環境中存活的潛力	☐	P
		監測	☐	P
		不良事件的應變計畫	☐	P
風險鑑定文件			☐	P

基因改造生物藥品所需風險評估文件

基因改造動物用生物藥品廠商自行審核書面報告

審查項目				自行審核	頁次
品質文件				☐	P
	用於生產種株的原始材料			☐	P
	構築載體使用的遺傳物質			☐	P
	載體			☐	P
	插入的核酸序列			☐	P
	基因工程疫苗最終產品的特性			☐	P
安全性及風險評估文件				☐	P
	危害鑑定			☐	P
		動物安全性		☐	P
			對象動物之安全性	☐	P
			非對象動物之安全性	☐	P
		公共衛生安全性		☐	P
		環境安全性		☐	P
		實驗設計		☐	P
		擴散和散播的潛力		☐	P
		在環境中存活的潛力		☐	P
		監測		☐	P
		不良事件的應變計畫		☐	P
風險鑑定文件				☐	P

風險評估文件:須包含品質和安全性/風險評估的詳細資料，惟第一類基改疫苗可以品質文件為主。

基因改造生物藥品所需風險評估文件

- 品質文件:
  - 用於生產種株的原始材料
  - 構築載體使用的遺傳物質
  - 載體
  - 插入的核酸序列
  - 基因工程疫苗最終產品的特性

基因改造生物藥品所需風險評估文件-5

說明：

1. 品質文件
   - A. 用於生產種株的原始材料：
     完整說明使用於生產種株的起始細胞、細菌、病毒和質體等所有材料。
   - B. 構築載體使用的遺傳物質：
     - a. 用於構築基因工程疫苗的質體，提供其構築、結構、序列及性質之詳細數據。
     - b. 使用於攜帶質體的細菌和重組質體的性狀和詳細起源，包含起動子（promoter）、促進子（enhancer）和外源基因序列，以及用於確認質體結構的分析方法等皆需提出資料。
     - c. 某些質體並不夠安定，如果會影響基因工程疫苗最終產品的穩定，則質體的不安定性必須加以排除。
     - d. 避免使用抗生素抗藥性基因為標記（marker），不能容許抗藥性基因轉移至基因工程疫苗最終產品。
   - C. 載體：
     - a. 說明有關構築基因工程疫苗的策略和基因改造的詳細資訊。
     - b. 載體的毒力基因必需加以確認，提供所剔除或加入之基因及其表達之蛋白質功能的資訊，詳細描述用於監測基因表現的標記。
     - c. 必需探討基因剔除對載體生物特性改變的影響。
     - d. 說明將質體基因序列插入載體的細節，以及探討載體插入基因對鄰近基因表現的影響。
     - e. 描述載體的遺傳特性，至少包括插入基因的部位及其兩側的基因序列的定序。
   - D. 插入的核酸序列：
     插入載體的核酸序列應使用適當的方法加以鑑定和定序。
   - E. 基因工程疫苗最終產品的特性：
     - a. 提出重組載體之基因型（genotype）和表現型（phenotype）的資訊，及其篩選和鑑別的方法。基因型和表現型的安定性、毒力、組織向性和宿主分佈等數據必需包含於安全性資料中。
     - b. 如果菌株在對象動物中無法複製，則必須於離體實驗使用一系列適當的對象動物細胞株加以確認。
     - c. 必須確認基因工程疫苗在製程中的安定性，以及插入的核酸序列不會發生重排或突變。
     - d. 基因工程疫苗表現的蛋白質的特性需以適當的生化、分生或免疫學方法加以鑑定，以確認最終產品的品質。
     - e. 需提供能區分原始載體和基因工程載體疫苗的技術方法。
2. 安全性/風險評估（risk assessment）

基因改造生物藥品所需風險評估文件

- 品質文件:
  - 構築載體使用的遺傳物質
    - 用於構築基因工程疫苗的質體,提供其構築、結構、序列及性質之詳細數據。
    - 使用於攜帶質體的細菌和重組質體的性狀和詳細起源,包含起動子(promoter)、促進子(enhancer)和外源基因序列,以及用於確認質體结構的分析方法等皆需提出資料。
    - 某些質體並不夠安定,如果會影響基因工程疫苗最終產品的穩定,則質體的不安定性必須加以排除。
    - 避免使用抗生素抗藥性基因為標記(marker),不能容許抗藥性基因轉移至基因工程疫苗最終產品。

基因改造生物藥品所需風險評估文件-1

http://163.29.152.42/Animal/

3

Blocking cells' antiviral response

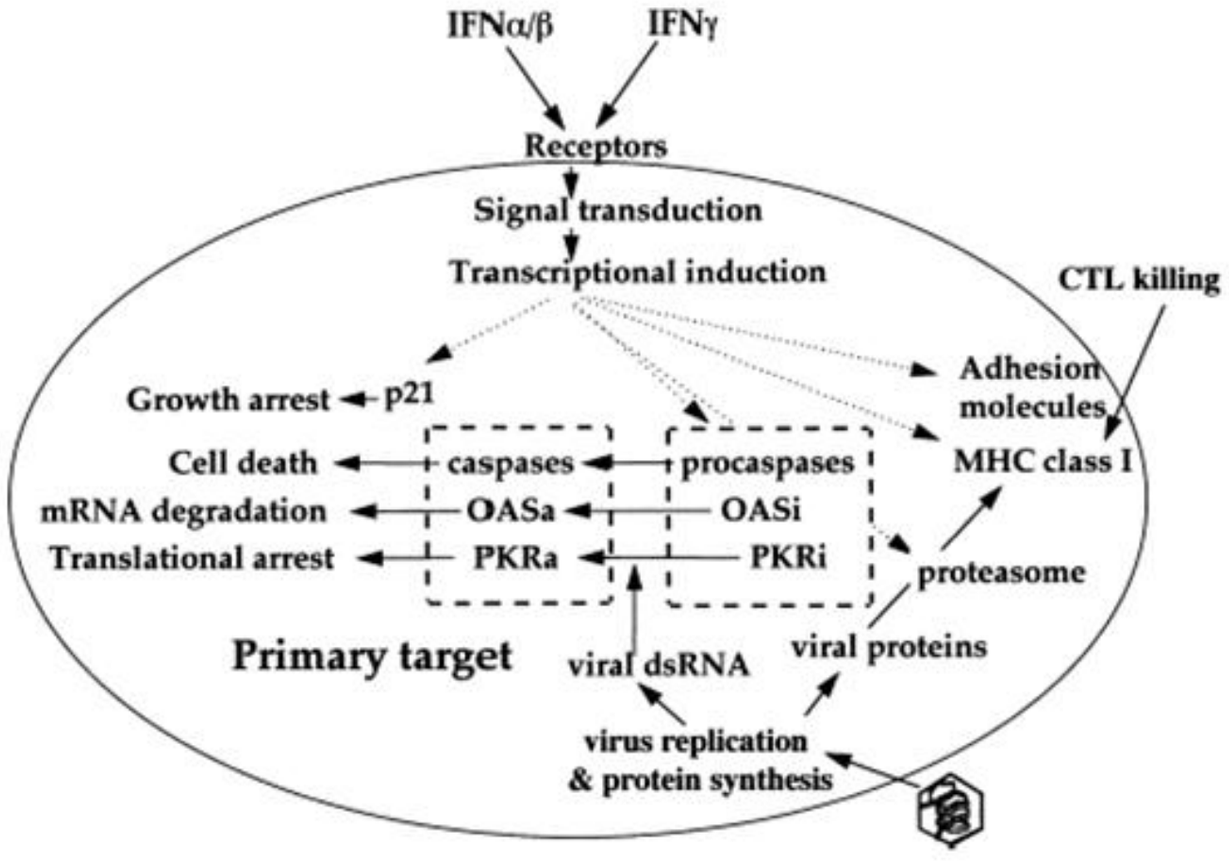


Table 1. Virus inhibition of IFN signalling and IFN-induced transcriptional responses

Virus	Mechanism of action/inhibition
i. Inhibition of IFN binding to cognate receptors	
Poxviruses (many)	Soluble IFN-α/β receptor
Poxviruses (many)	Soluble IFN-γ receptor
ii. Inhibition of Jak/STAT pathway	
Adenovirus	E1A decreases the levels of STAT1 and p48; sequesters the transcriptional co-activator, CBP/p300, which binds STAT1 and STAT2; interacts directly with STAT1
Ebola virus	Blocks IFN-α/β and IFN-γ signalling, mechanism unknown
Epstein–Barr virus	EBNA-2 blocks IFN signal transduction, mechanism unknown
Hepatitis C virus	Blocks IFN-α/β and IFN-γ signalling, mechanism unknown
Human cytomegalovirus	Reduces levels of Jak1 and p48
Human parainfluenza virus type 2	Blocks IFN-α/β signalling by targetting STAT2 for degradation
Human parainfluenza virus type 3 and Sendai virus	Block IFN-α/β and IFN-γ signalling by blocking STAT1 phosphorylation
Human papillomavirus type 16	E7 protein binds to p48 and blocks IFN-α/β signalling
Murine polyoma virus	T antigen binds to and inactivates Jak1
Simian virus 5 (and mumps virus?)	V protein blocks IFN-α/β and IFN-γ signalling by targetting STAT1 for proteasome-mediated degradation
iii. Miscellaneous	
Hepatitis B virus	Capsid protein specifically inhibits MxA gene expression, mechanism unknown
Human herpesvirus-8	Virus IRF homologue blocks transcriptional responses to IFN-α/β and IFN-γ

(Goodbourn *et al*., 2000)

Table 2. Virus inhibition of IFN-induced antiviral enzymes

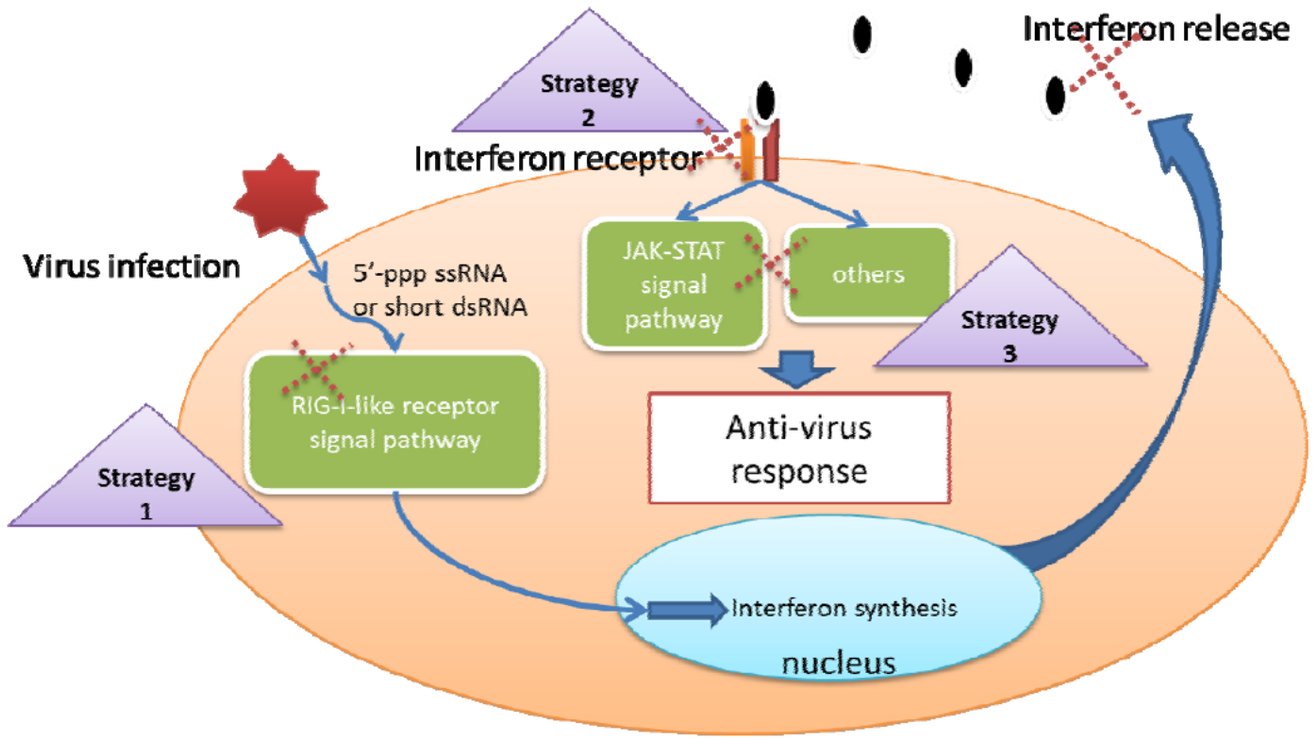
Virus	Mechanism of action/inhibition
i. PKR	
Adenovirus	Produces VA RNA that binds to but fails to activate PKR
Baculovirus	PK2 binds eIF2α kinases, including PKR, and blocks their activities
Epstein–Barr virus	Produces EBER RNA that binds to but fails to activate PKR
Hepatitis C virus	NS5A binds to and inhibits PKR; E2 also interacts with PKR and may inhibit its activity
Herpes simplex virus	ICP 34.5 redirects protein phosphatase 1 to dephosphorylate (re-activate) eIF2α; U_s11 blocks PKR activity
Human immunodeficiency virus	Down-regulates PKR by unknown mechanism; Tat and short Tat-responsive region RNA inhibit PKR
Influenza virus	NS1 binds dsRNA and PKR to inhibit its activity. Influenza virus also induces cellular inhibitor of PKR (p58IPK)
Poliovirus	Induces the degradation of PKR
Poxviruses (many)	Example: vaccinia virus E3L binds dsRNA and PKR; K3L binds PKR
Reovirus	σ3 binds dsRNA and thus inhibits PKR (and 2′–5′ oligoadenylate synthetase)
Rotavirus	NSP3 binds dsRNA and thus inhibits PKR (and 2′–5′ oligoadenylate synthetase)
ii. 2′–5′ Oligoadenylate synthetase/RNase L system	
Various viruses	Produce proteins that sequester dsRNA (above)
Encephalomyocarditis virus	Induces RNase L inhibitor (RLI) that antagonizes 2′5′A binding to RNase L
Herpes simplex virus	2′5′A derivatives are synthesized that behave as 2′5′A antagonists
Human immunodeficiency virus	Induces RNase L inhibitor (RLI) that antagonizes 2′5′A binding to RNase L

(Goodbourn *et al*., 2000)

高敏感細胞研發策略

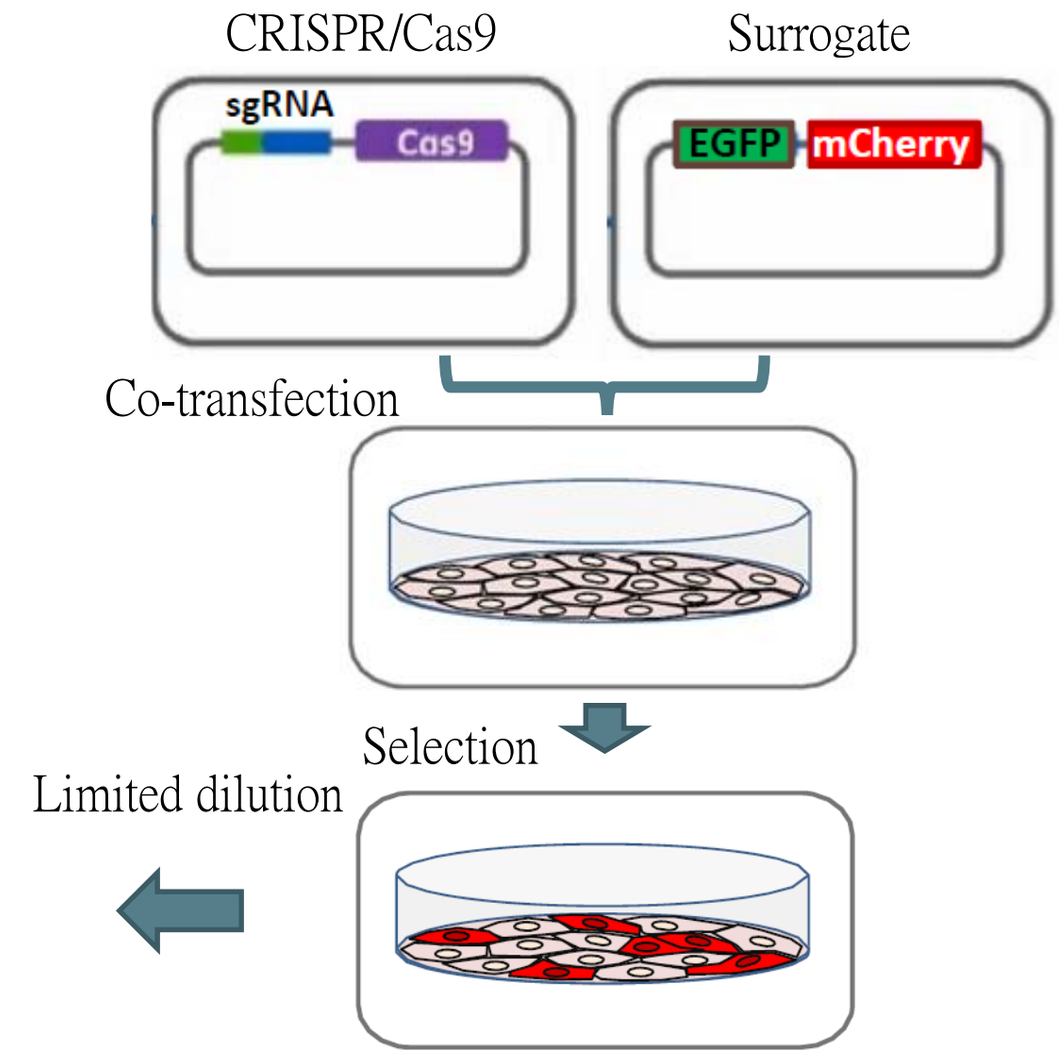
1. 運用分子生物學基因編輯技術CRISPR/Cas9，抑制多種可能干擾病毒複製的IFN 系統功能，剔除生產用細胞株之干擾素受體相關基因。
2. 細胞轉染病毒抑制細胞先天免疫蛋白基因。
3. 細胞自然突變選殖。

藉以達成穩定增產病毒性抗原之目的



Cells-IFNabR1 cell line

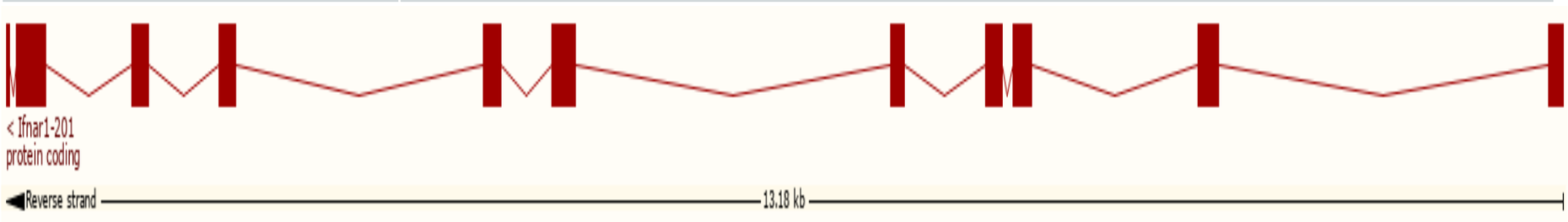
- BHK21 cell line and sgRNA design will send to Sinica
- IFNabR1 knock-out processes will entrusted to Sinica



Single cell clone selection

Interferon alpha and beta receptor subunit 1 (IFNabR1)

Item	Description
Name	interferon (alpha and beta) receptor 1 [Source:MGI Symbol;Acc:MGI:107658]
Gene Synonyms	IFN-alpha/betaR, Ifar, Ifrc
Location	Scaffold KB708169.1: 6,405,709-6,418,891 reverse strand.
About this transcript	This transcript has 11 exons and is annotated with 20 domains and features
Gene	This transcript is a product of gene ENSMAUG00000014001 Hide ranscript table



Sequence contain 11 exons, 10 introns, 5' upstream sequence and 3' downstream sequence

IFNabR1 transcription domain

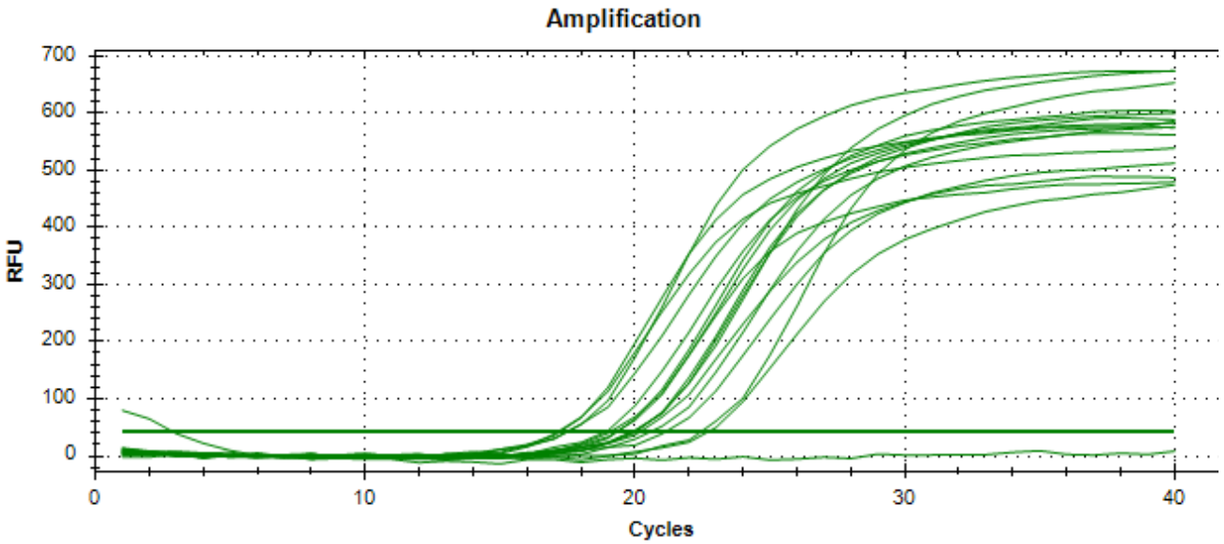
- ✓ IFNabR1 transcript is annotated with domains and features.
- ✓ Select the Interferon/interleukin receptor domain

Domain source	Start	End	Description
Pfam	2	89	Fibronectin type III
Smart	4	87	Fibronectin type III
Superfamily	5	110	Fibronectin type III
Prosite_profiles	6	101	Fibronectin type III
Superfamily	86	198	Fibronectin type III
Prosite_profiles	102	201	Fibronectin type III
Smart	103	187	Fibronectin type III
Smart	204	386	Fibronectin type III
Superfamily	205	314	Fibronectin type III
Superfamily	307	399	Fibronectin type III
Prosite_profiles	307	402	Fibronectin type III
Gene3D	2	201	Immunoglobulin-like fold
Gene3D	202	403	Immunoglobulin-like fold
Pfam	102	199	Interferon/interleukin receptor domain
Pfam	305	399	Interferon/interleukin receptor domain

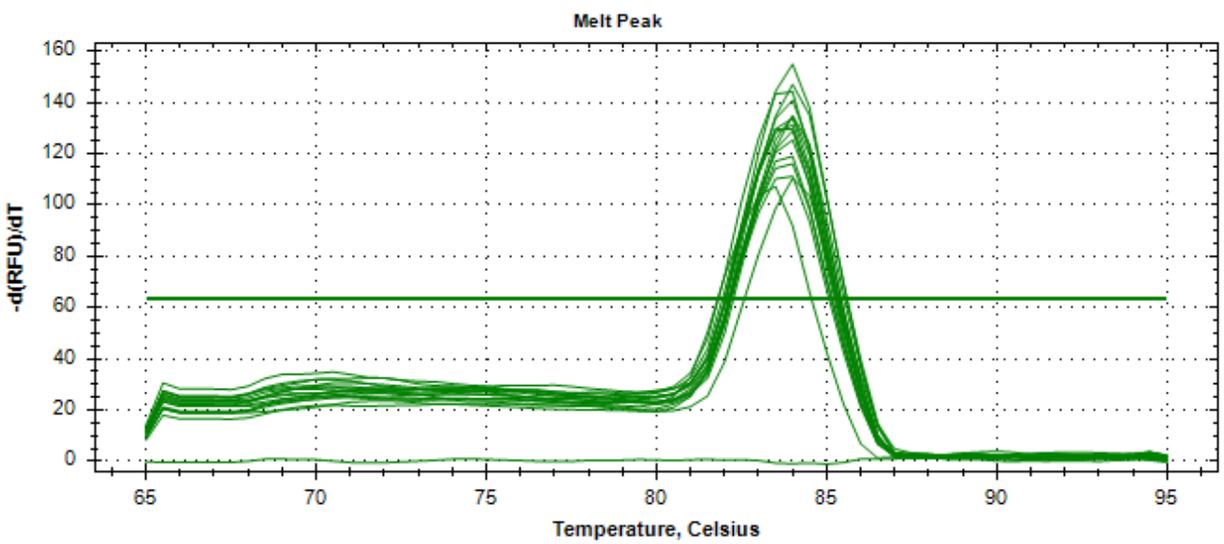
IBDV感染不同BHK21之病毒增殖情形

Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct
1	20.21	6	22.46	11	20.6	16	20.3	21	22.34
2	22.13	7	22.72	12	18.94	17	22.1	Positive control	16.61
3	19.11	8	22.74	13	18.3	18	22.52	Parent Cell	22.13
4	21.64	9	19.36	14	19.91	19	19.33		
5	21.15	10	19.97	15	20.06	20	22.8		

圖、IBDV生長於不同BHK21克隆後進行qPCR定量分析，可見BHK21-12及BHK21-13對於IBDV具最佳之感受性，病毒增殖力價明顯高於原始BHK21細胞。



圖、IBDV生長於不同BHK21克隆後進行qPCR定量分析

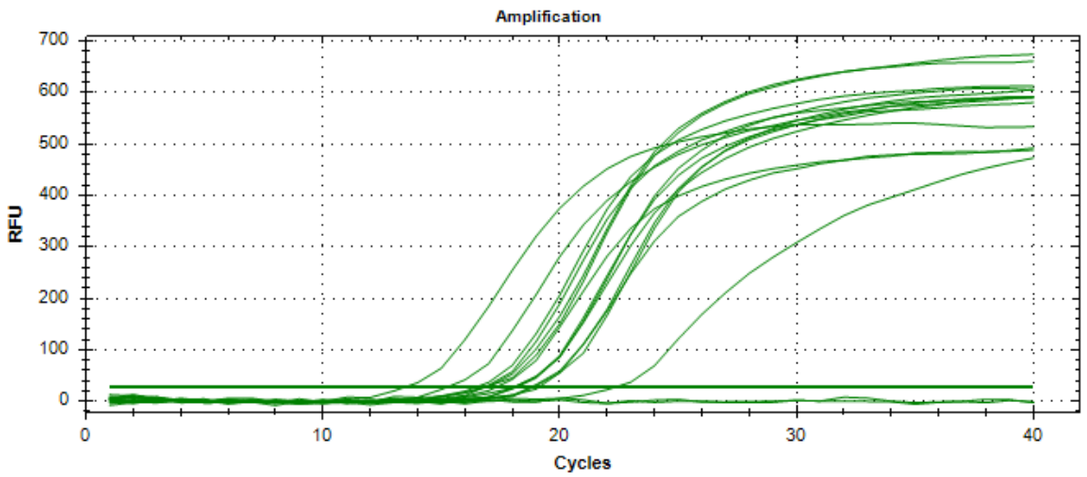


圖、 IBDV生長於不同BHK21克隆後進行qPCR熔點分析

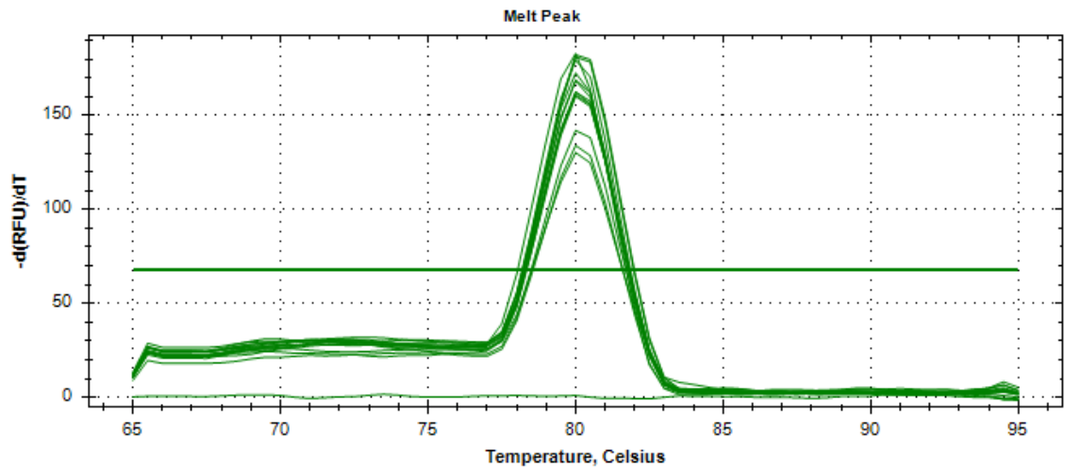
PEDV感染不同BHK21之病毒增殖情形

Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct	Cells NO.	Ct
1	18.83	6	16.75	11	18.13	16	16.77	21	17.58
2	19.89	7	18.63	12	17.28	17	16.42	Positive control	13.21
3	22.28	8	19.09	13	15.2	18	18.24		
4	19.53	9	20.5	14	22.6	19	20.6		
5	18.03	10	18.04	15	21.9	20	18.73		

圖、PEDV生長於不同BHK21克隆後進行qPCR定量分析，可見BHK21-13對於PEDV具最佳之感受性



圖、PEDV生長於不同BHK21克隆後進行qPCR定量分析



圖、 PEDV生長於不同BHK21克隆後進行qPCR熔點分析

不同IBDV病毒株感染BHK21細胞後之病毒增殖情形

Strain	Virus Titer(Ct)		
	BHK21	BHK21-12	BHK21-13
2512	22.21	18.33	18.45
Luker	21.83	17.94	18.76
Medivac	25.12	21.32	21.42

表、不同IBDV生長於不同BHK21克隆後進行qPCR定量分析，可見BHK21-13對於IBDV比原始BHK21具較好之感受性，病毒增殖力價明顯高於原始BHK21細胞。不同PEDV包括CV777株、TW14-1株、TW14-2株皆有相同之結果。

不同PEDV病毒株感染BHK21細胞後之病毒增殖情形

Strain	Virus Titer(Ct)	
	BHK21	BHK21-13
CV777	19.21	15.45
TW14-1	24.12	20.21
TW14-2	25.83	19.42

表、不同PEDV生長於BHK21-13克隆後進行qPCR定量分析，可見BHK21-12及BHK21-13對於IBDV比原始BHK21具較好之感受性，病毒增殖力價明顯高於原始BHK21細胞。不同IBDV包括2512株、Luker株、Medivac株皆有相同之結果。

IBD luker live vaccine safety test

Animal NO.	Titera (Log 10)	Bursa	Body	BBR	Mean
1	5.5	0.61g	124.65g	4.89	4.16
2	5.5	0.42g	145.24g	2.89	
3	5.5	0.65g	145.21g	4.48	
4	5.5	0.81g	182.96g	4.43	
5	5.5	0.7g	156.73g	4.47	
6	5.5	0.75g	175.12g	4.28	
7	5.5	0.43g	115.76g	3.71	
8	5.5	0.36g	117.14g	3.07	
9	5.5	0.78g	149.05g	5.23	
Control-1		0.8g	151.35g	5.29	4.84
Control-2		0.57g	129.91g	4.39	

Table. The bursa-body weight ratio (BBR) of the safety test in 1-day-old SPF chickens immunized with IBD-Luker attenuated in BHK21-13 cell.

BBR: Bursa/Body*1000

IBD 活毒疫苗血清抗體中和試驗

Titer/dose TCID50	VN Titer					
	1w	**2w**	**3w**	**4w**	**5w**	**6w**
10^4.5	4	32	64	128	128	256
10^5.5	32	256	512	1024	1024	1024
Control	< 2	< 2	< 2	< 2	< 2	< 2

- 經干擾素受體基因剃除之 BHK21細胞其中BHK21-12及BHK21-13細胞對家禽傳染性華氏囊炎病毒之感受性比原始細胞明顯提升。而BHK21-13細胞對豬流行性下病毒之感受性比原始細胞明顯提升。

- 以BHK21-13馴化IBD-Luker株後，以1日齡SPF雞隻免疫$10^{5.5}TCID_{50}$病毒測其BBR、BGL、BHL等安全性指標結果顯示BHK21-13培養之IBD-Luker病毒其對雞隻華氏囊傷害極輕微。

- 以BHK21-13馴化IBD-Luker株後，以14日齡SPF雞隻免疫$10^{5.5}TCID_{50}$病毒，免役後每週採血觀察6週，測其血清中和抗體，結果顯示中和抗體2週後後可達256倍，4週後後可達1024倍，並維持至6週以上。

不同PEDV病毒株感染1日齡豬隻後之發病情形

	D1	D2	D3	D4	D5	D6	D7
CV777	N	N	N	N	N	N	N
TW14-1	N	N	N	Di	Di	De	-
TW14-2	N	N	N	N	Di	Di	De

N: Normal
De: Dead
Di: Diarrhea

- 經干擾素受體基因剃除BHK21-13細胞對豬流行性下病毒之感受性比原始細胞明顯提升。

- 以BHK21-13細胞培養之PEDV野外強毒株口服給予1日齡豬隻可成功誘發下痢性疾病並造成死亡，可見其病毒株仍保有完整毒力。

V protein of Paramyxovidae

Virus	Mechanism of inhibition	Note
NDV	Degradation of STAT1	B1 straine
SV5	Degradation of STAT1	
HPIV2	Degradation of STAT2	
Nipah Virus	Inhibition of STAT1/2 function	
Hendra virus	Inhibition of STAT1/2 function	
Measles virus	Inhibiting STAT1/2 phosphorylation	
Sendai virus	Inhibiting STAT1/2 phosphorylation	

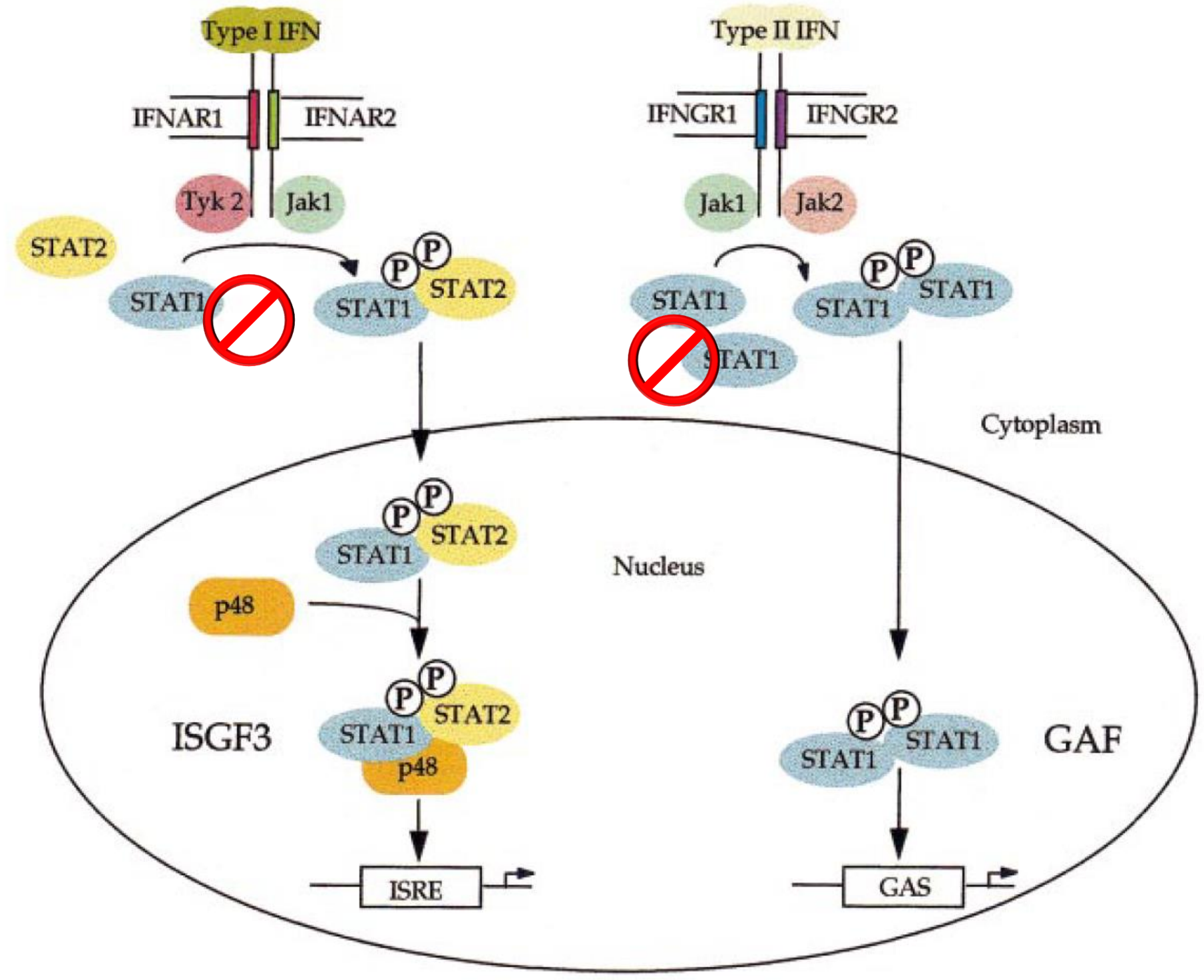


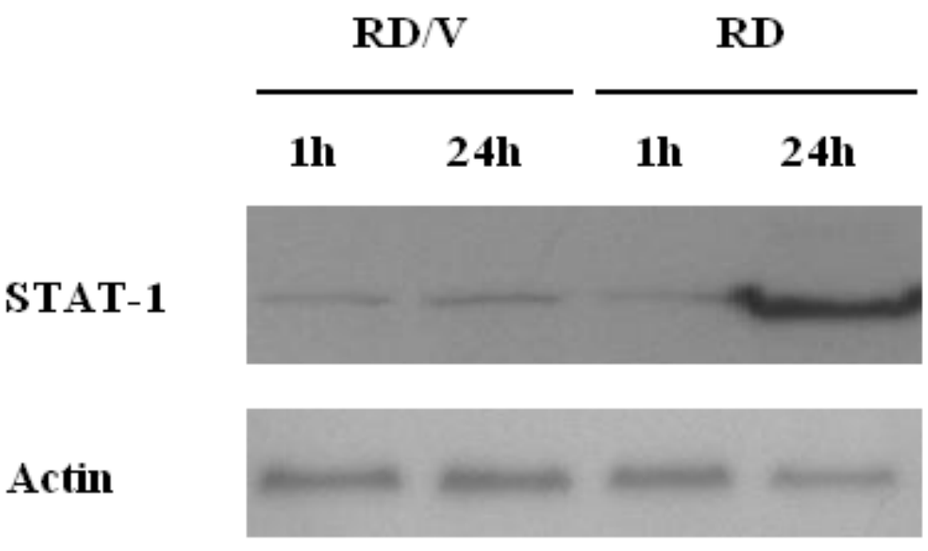
Fig. 3. Signalling pathways activated by IFN-α/β and IFN-γ. The biological activities of IFNs are initiated by binding to their cognate receptors. This leads to the activation of receptor-associated tyrosine kinases, as discussed in the text. These kinases phosphorylate members of the STAT family of transcription factors, which can enter the nucleus and, either on their own or in combination with p48, bind to the promoters of target genes and bring about gene-specific transcriptional activation. See text for details.

IFN α / β

Cell

Cell-V

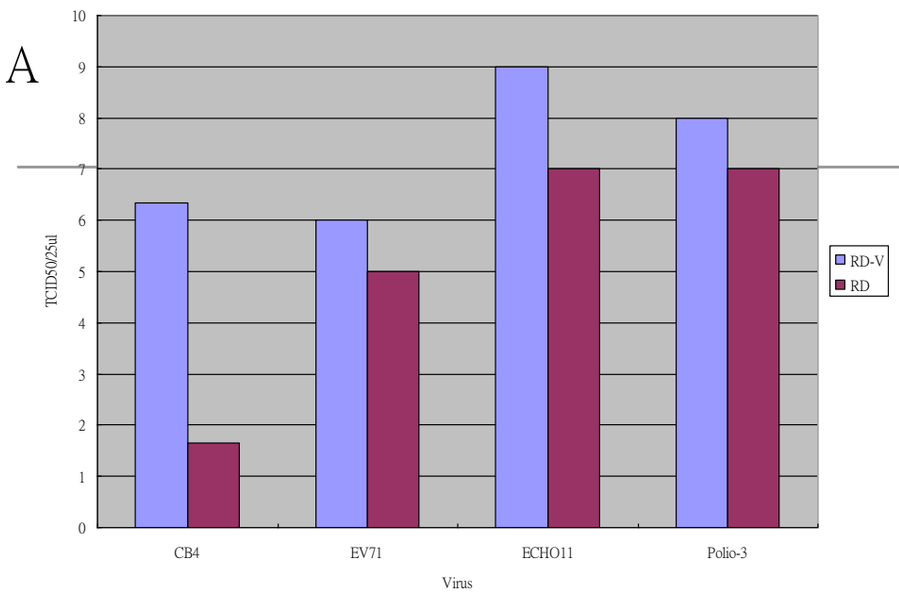
Western blot

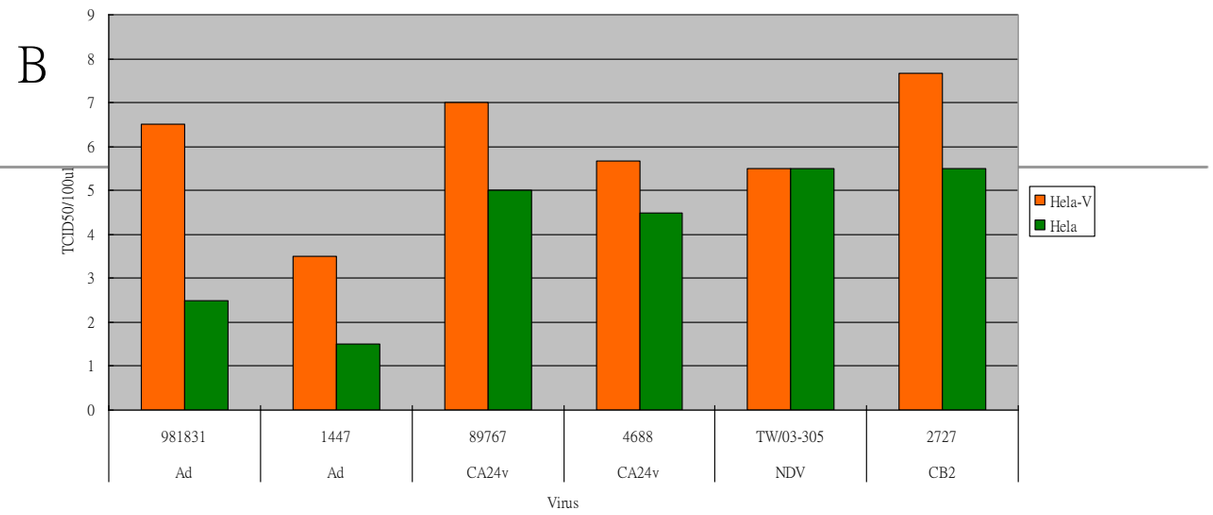


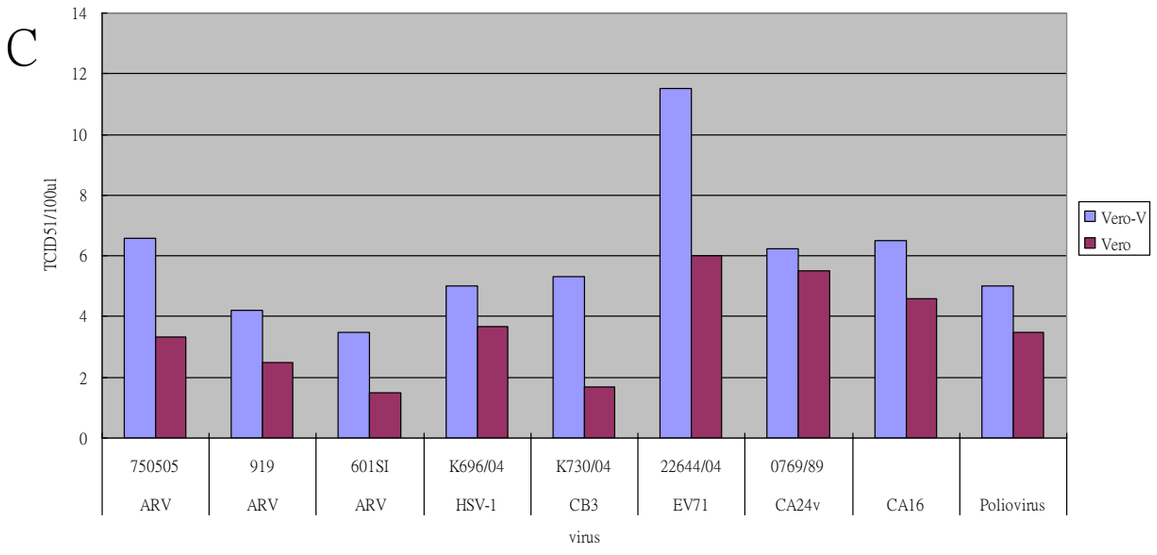
STAT1

Phospho-STAT1

STAT2







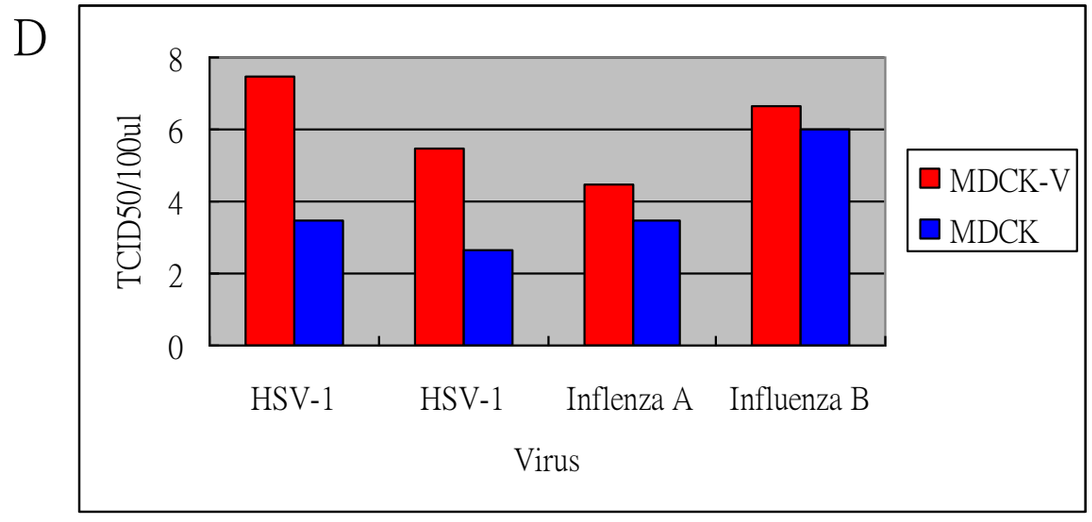
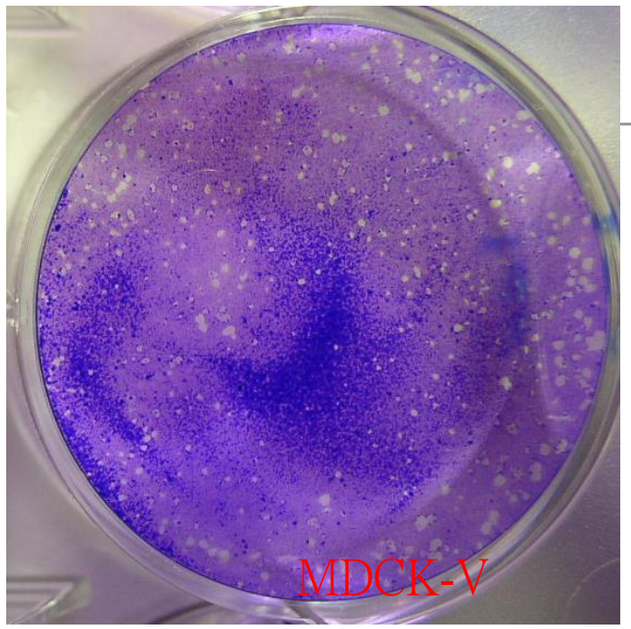
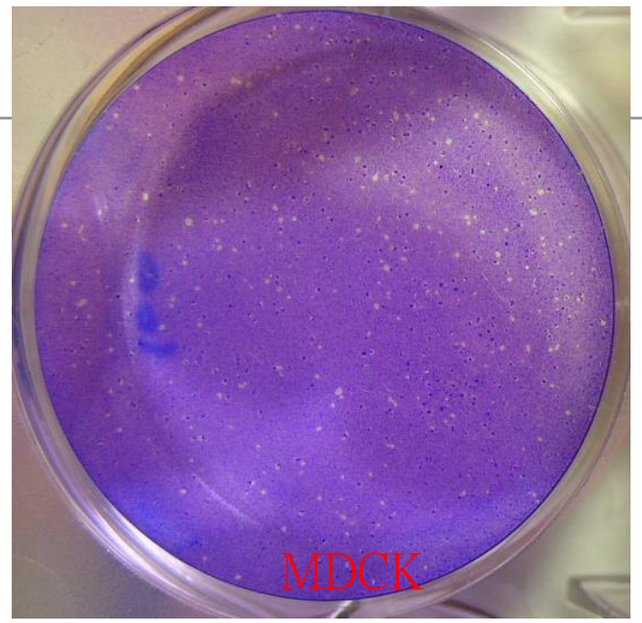
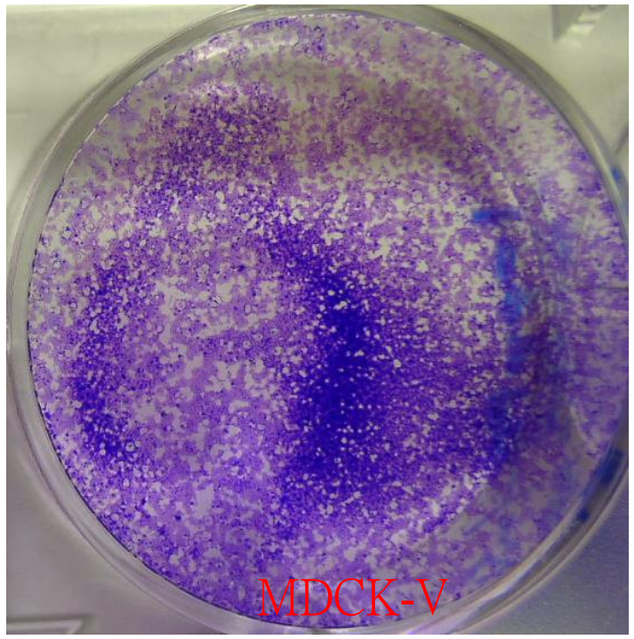


Figure. 比較病毒於(A)RD 細胞及本技術平台處理之RD 細胞(RD-V) (B) HeLa 細胞及本技術平台處理之HeLa 細胞(HeLa-V) (C)vero 細胞及本技術平台處理之vero 細胞(vero-V) (D)MDCK 細胞及本技術平台處理之MDCK 細胞(MDCK-V)產生之病毒力價差異。







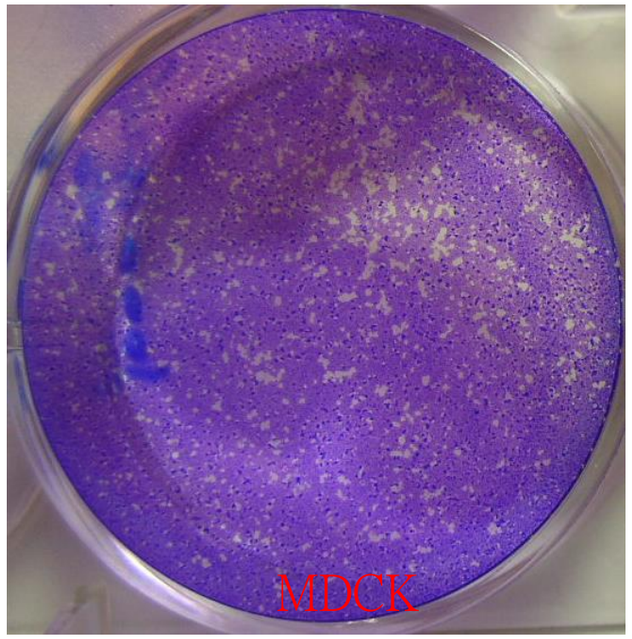


Fig. Comparison of plaque formation in MDCK and MDCK-V cells infected with 100 PFU of HSV-1.

Plaque Size?

Vero-V

Vero

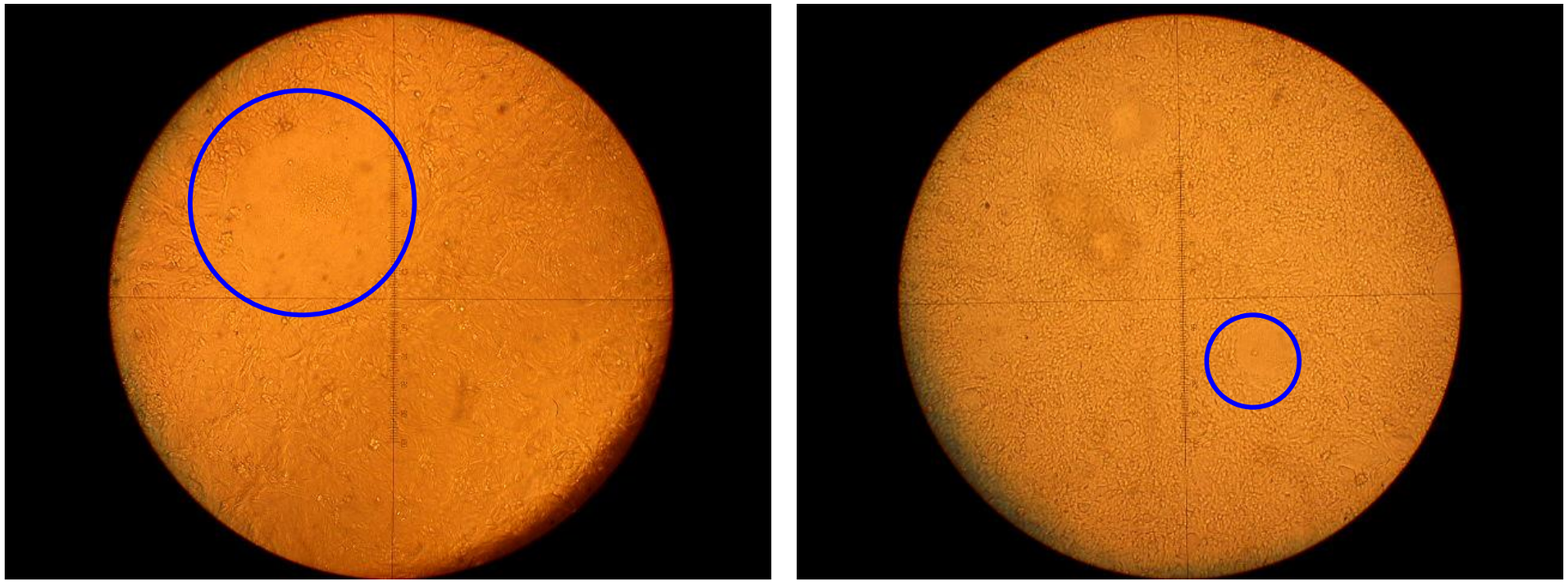
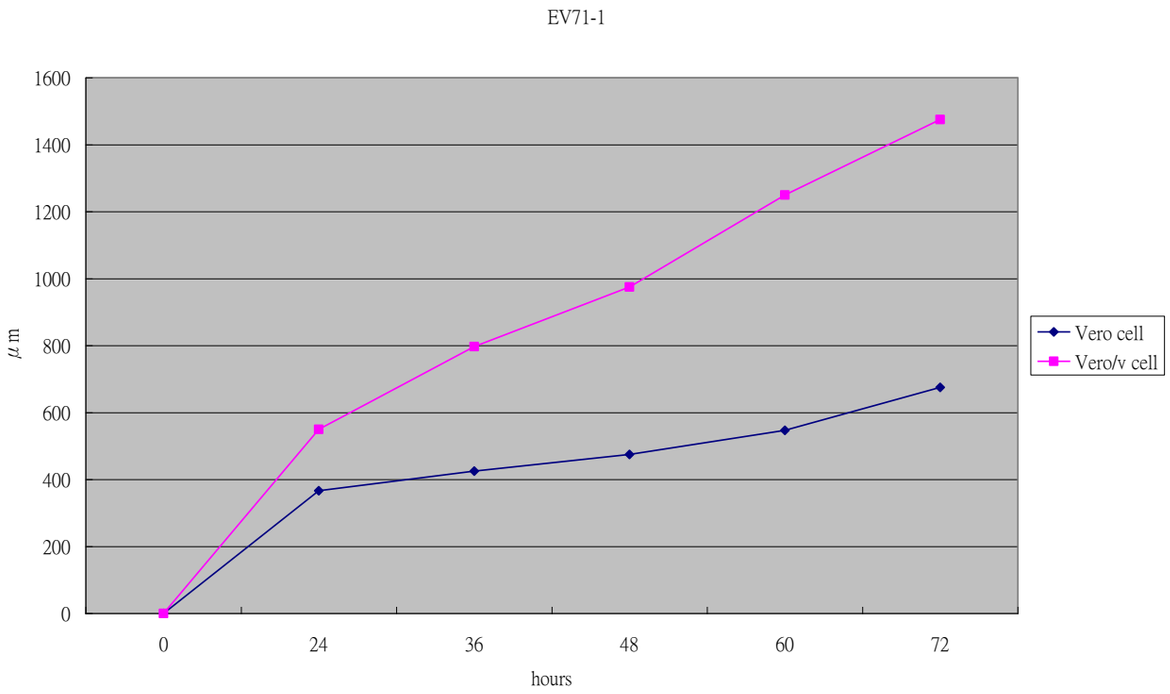
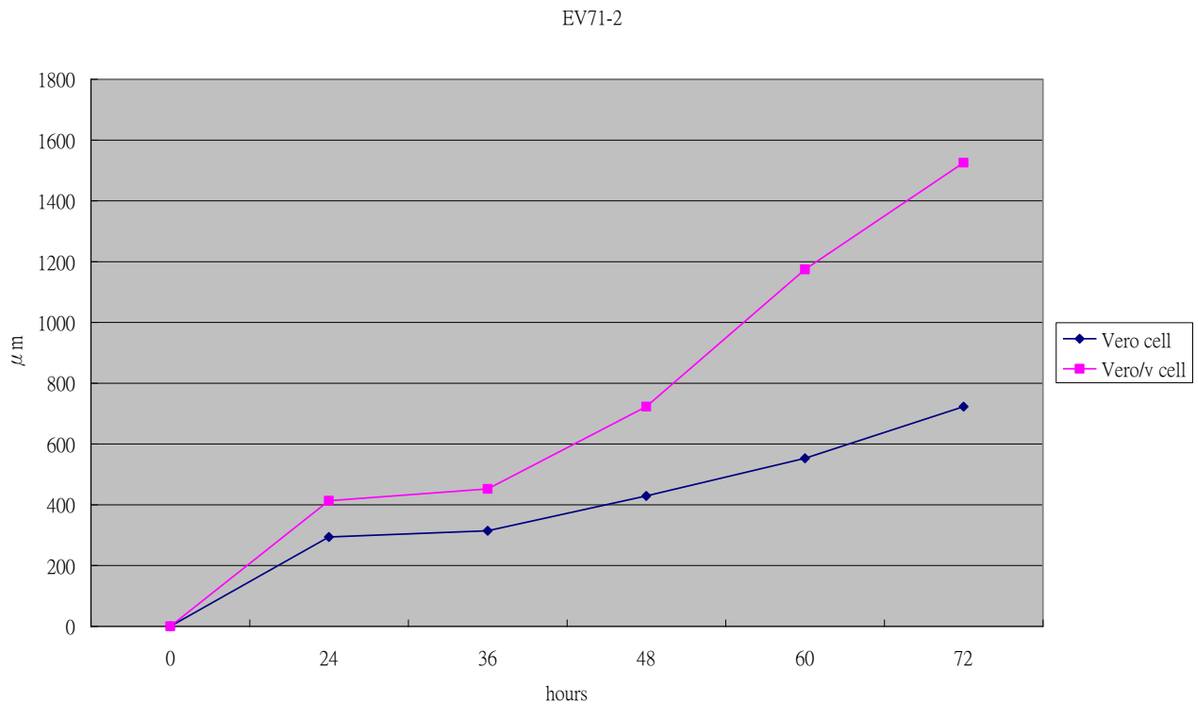
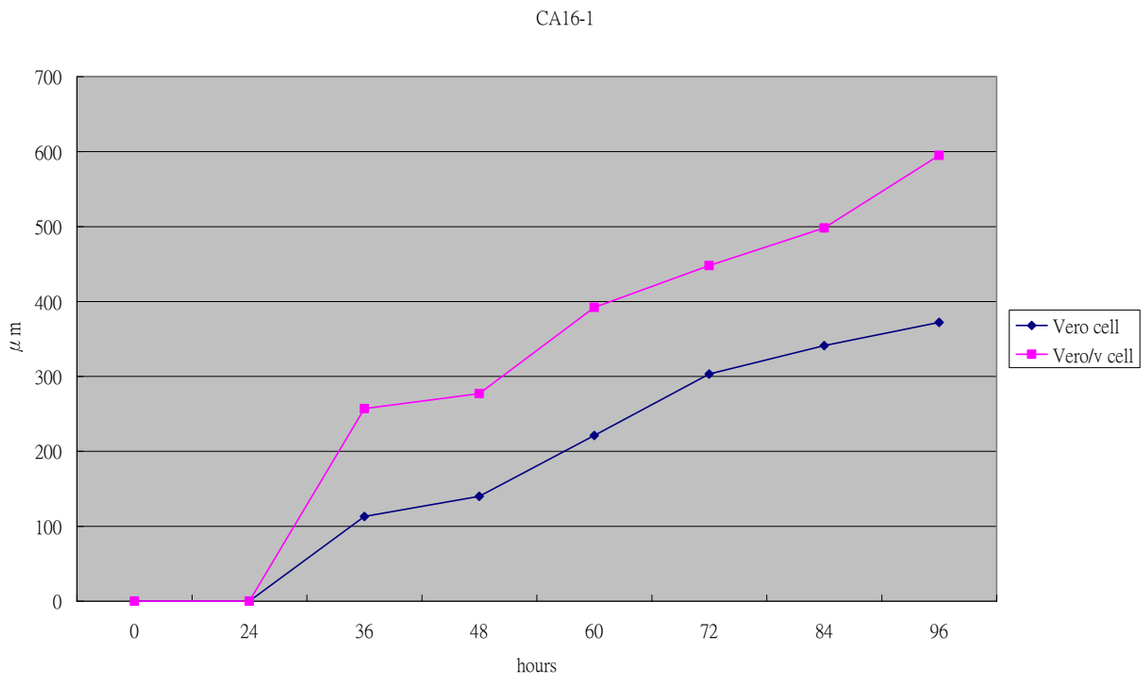


Fig. Comparison of plaque formation in Vero and Vero-V cells infected with 100TCID50 of ARV(750505) .

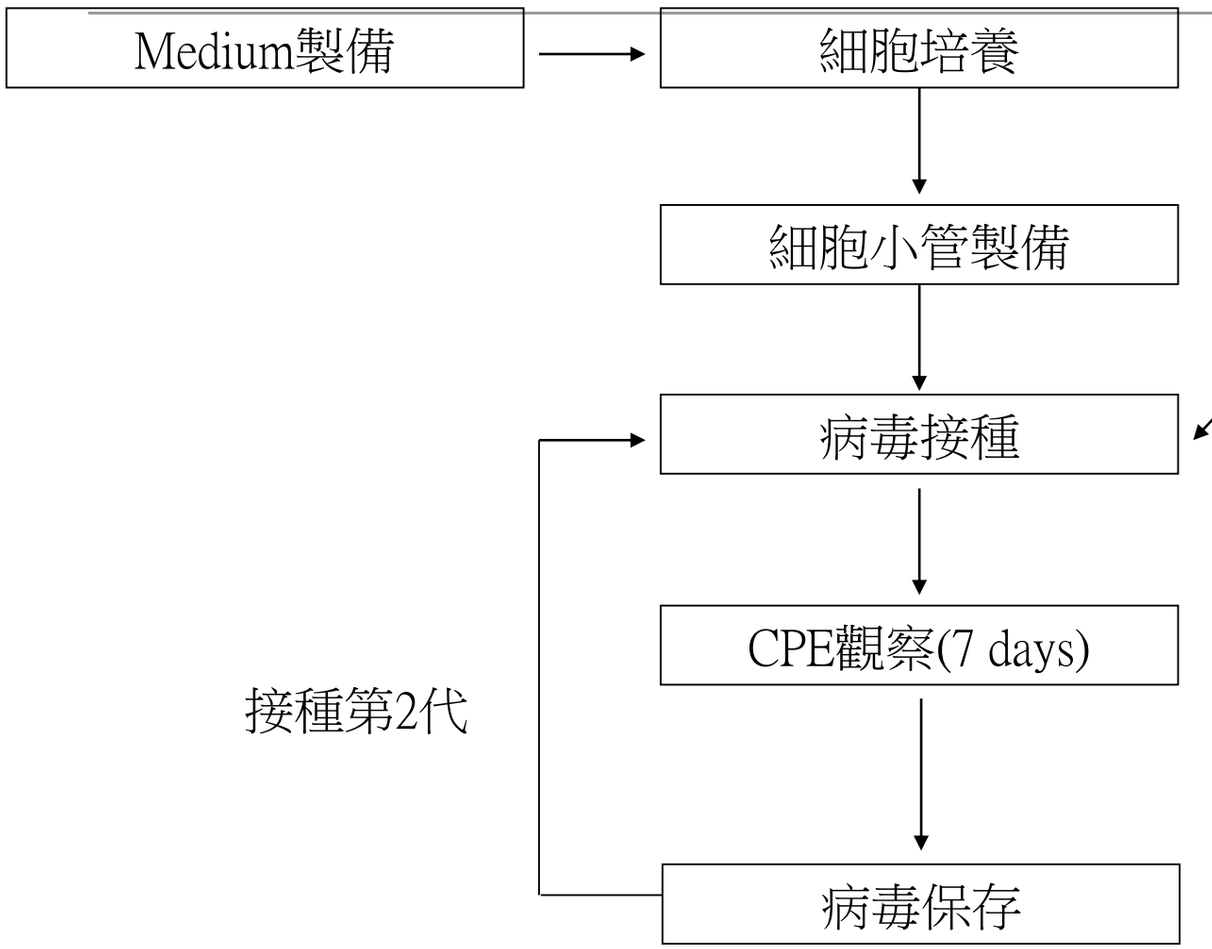
Plaque Size?

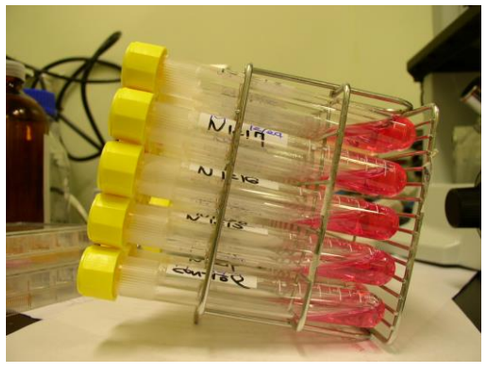

EV71-1
1600
1400
1200
1000
800
600
400
200
0
μm
0
24
36
48
60
72
hours
Vero cell
Vero/v cell


EV71-2
1800
1600
1400
1200
1000
800
600
400
200
0
μm
0
24
36
48
60
72
hours
Vero cell
Vero/v cell


CA16-1
700
600
500
400
300
200
100
0
μm
0
24
36
48
60
72
84
96
hours
Vero cell
Vero/v cell

病毒分離操作流程





Cells	Date/ Positive NO.										Total Positive	Positive Rate (%)
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
RD-V	3	2	0	6	0	2	7	5	1	0	26	36
RD	0	0	1	4	0	1	3	1	0	0	10	14

Table.72個泡疹性咽峽炎檢體病毒分離情形

病毒馴化

病毒

細胞株

劇烈突變

死亡

不易存活/失去抗原性?

病毒

基因工程細胞

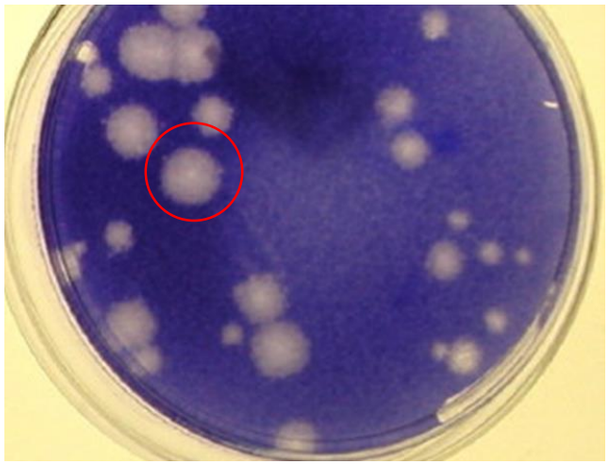
突變

死亡

容易存活/
保留抗原性

外源基因
檢測

生產用
細胞株

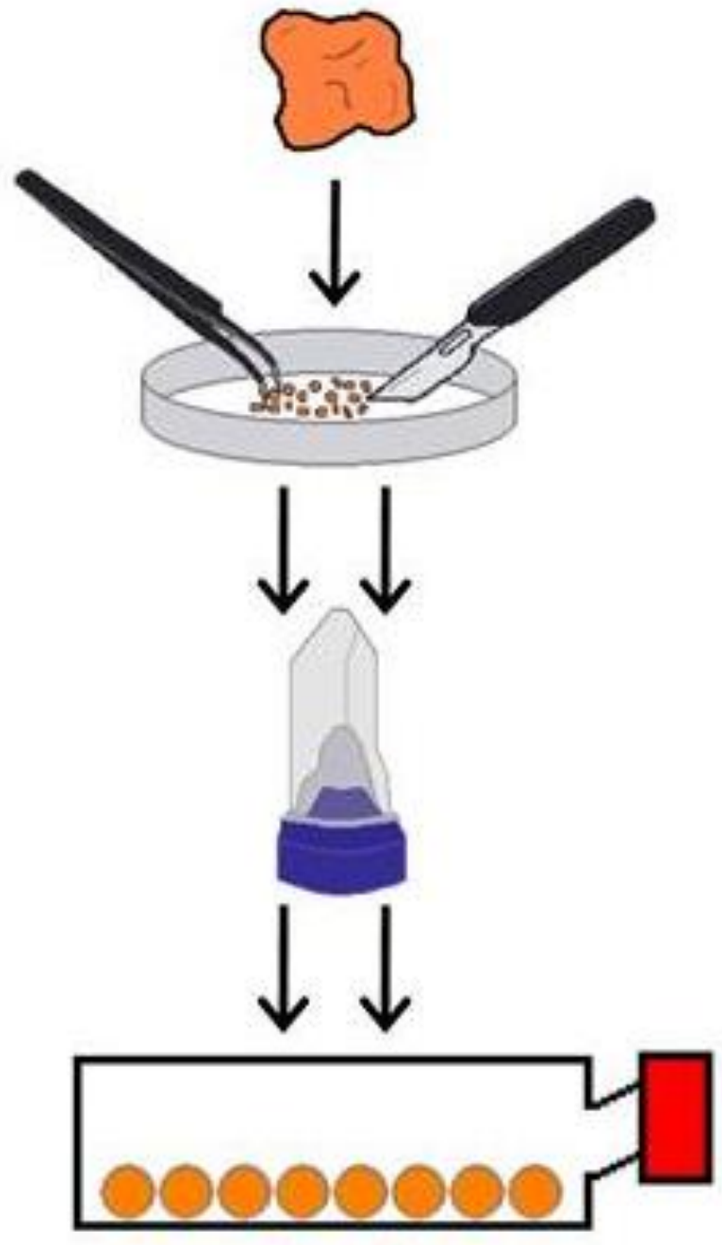


選殖

連續繼代

4

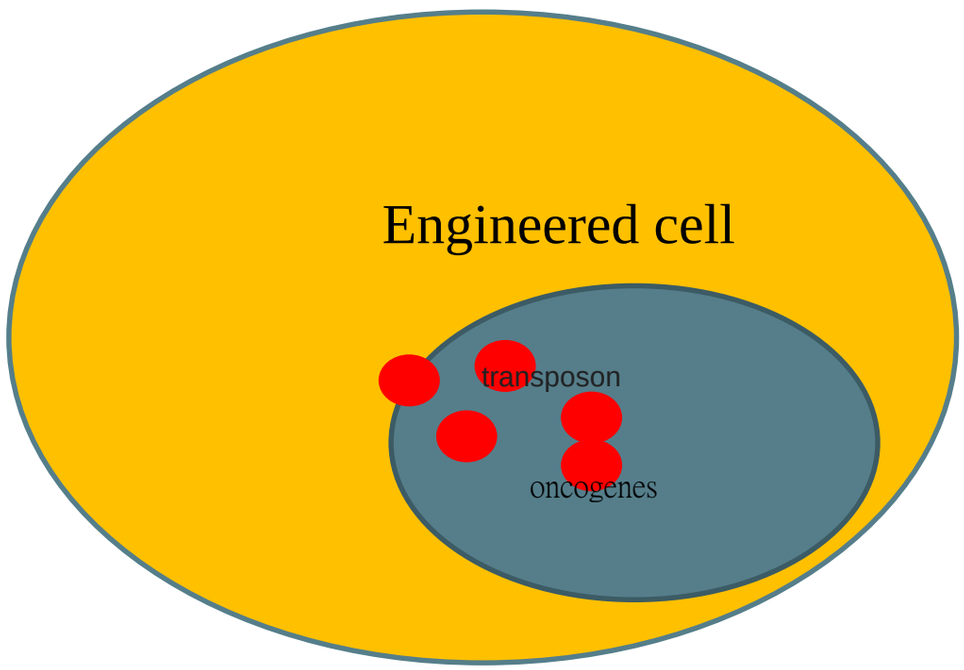
Immortalization of primary cells

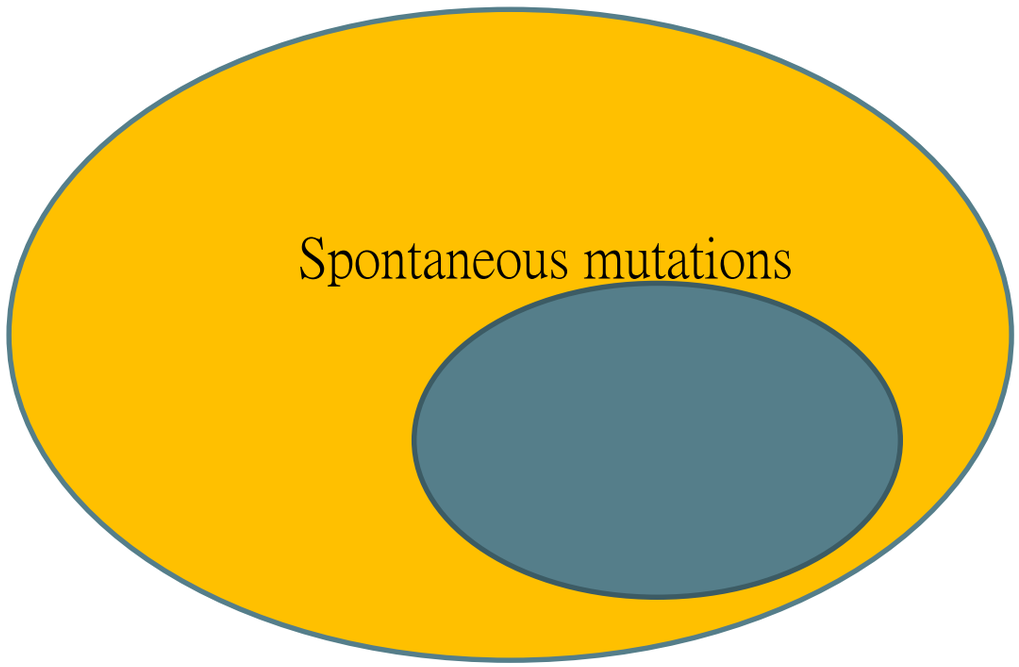


Principles

- Certain viruses have strict requirement for host cells.
- We can obtain primary cells from the specific host, but immortalization of those primary cells is needed for continuous virus production.
- Immortalization methods include:
  - Introducing oncogenes
  - spontaneous mutations
  - Viral infections

Benefit of Spontaneous mutations?





Biosecurity? GMO specification? Cells stability?
Antibiotic resistance gene? .....

Mass production·····

永生化细胞系的建立

- 病毒適應細胞能力與病毒自然宿主、病毒原發器官和細胞類型有關。

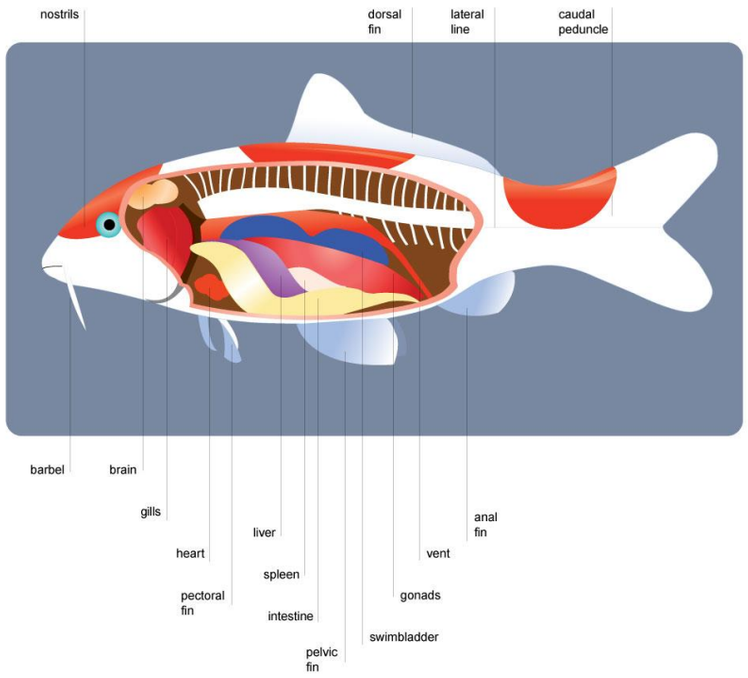
- 很多病毒宿主範圍很窄，只有適宜宿主細胞才能複製，釋放子代病毒。

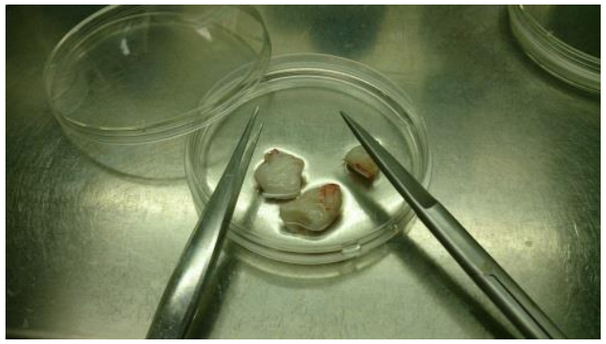
- 細胞永生化是指體外培養的細胞經過自發的或受外界因素的影響從增殖衰老危機中逃離，從而具有無限增殖能力的過程。

細胞永生化策略

- 病毒轉染(EB病毒、SV40病毒、乳頭瘤病毒E6 、肝炎病毒、腺病毒E1)
- 端粒酶
- 放射線(X射線、電離輻射、Co60)
- 癌基因、原癌基因
- 自然突變

Primary culture





dissected tissue

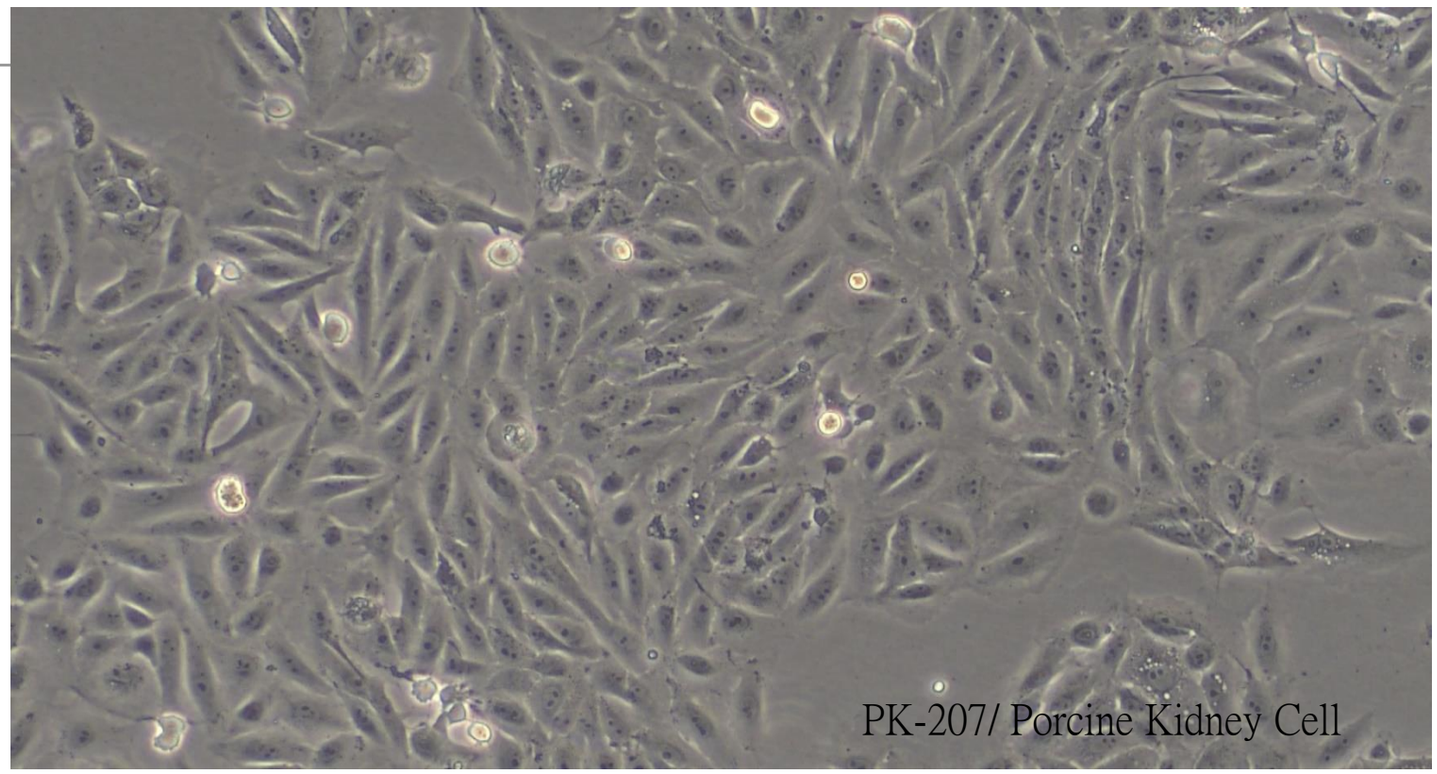
PBS wash

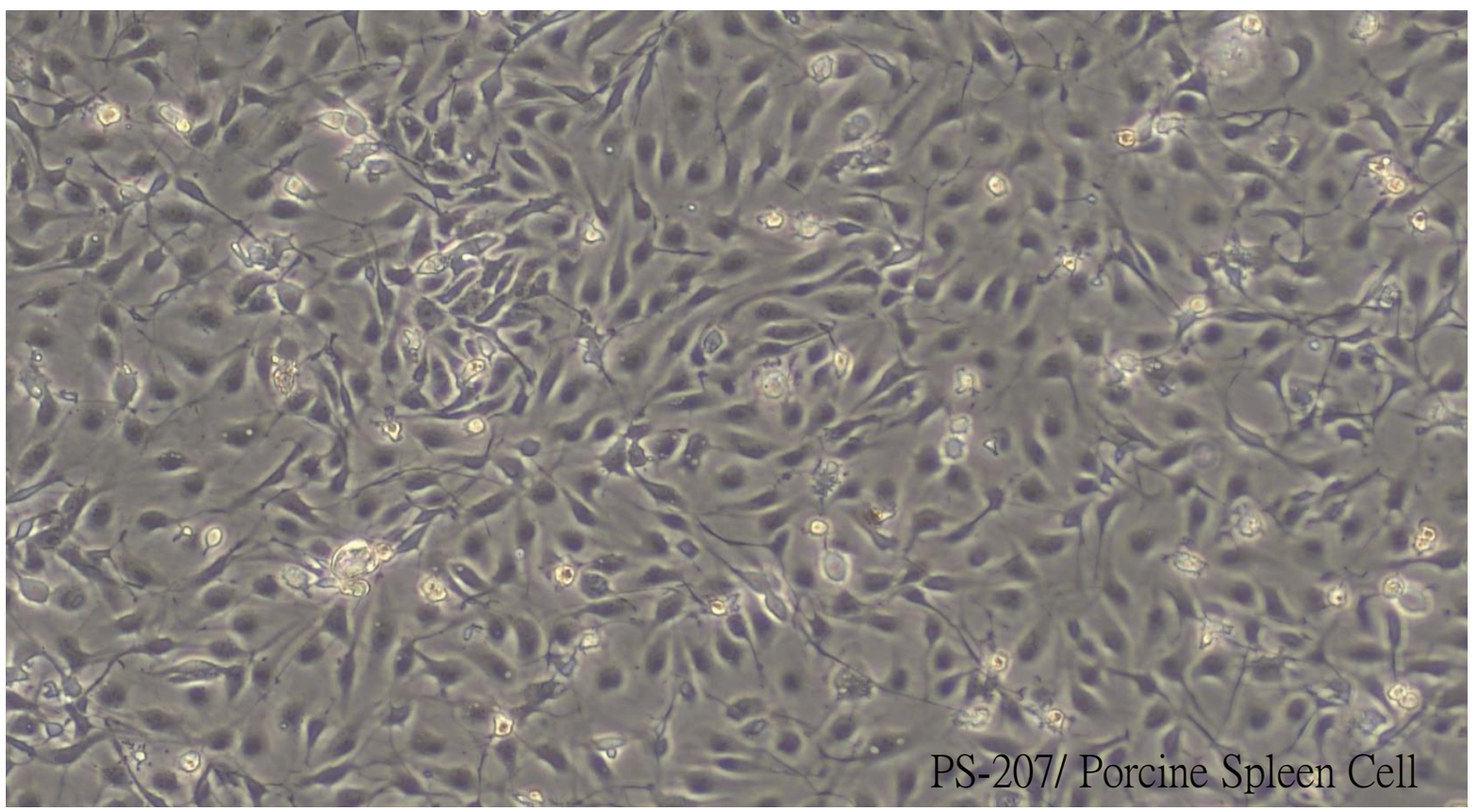
digested with trypsin

1000rpm,10min

25℃
incubation

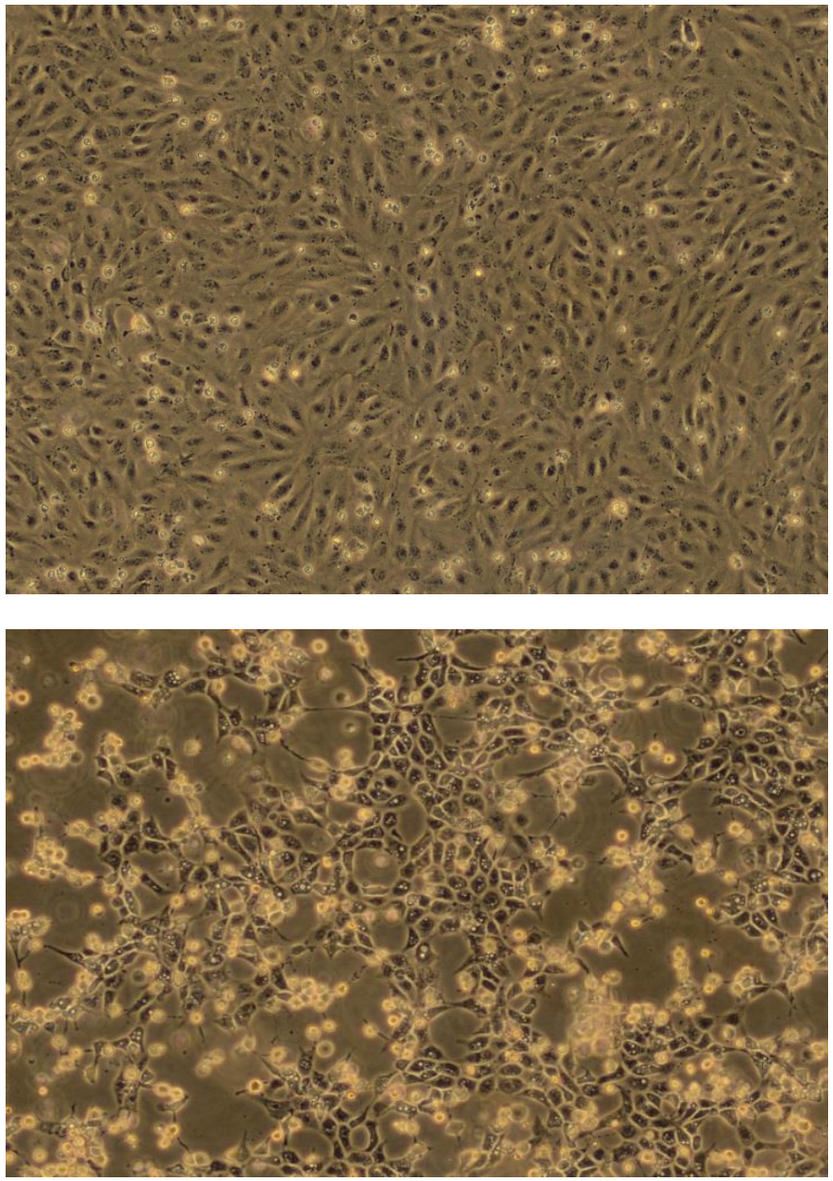
Establishment of Swine cell line





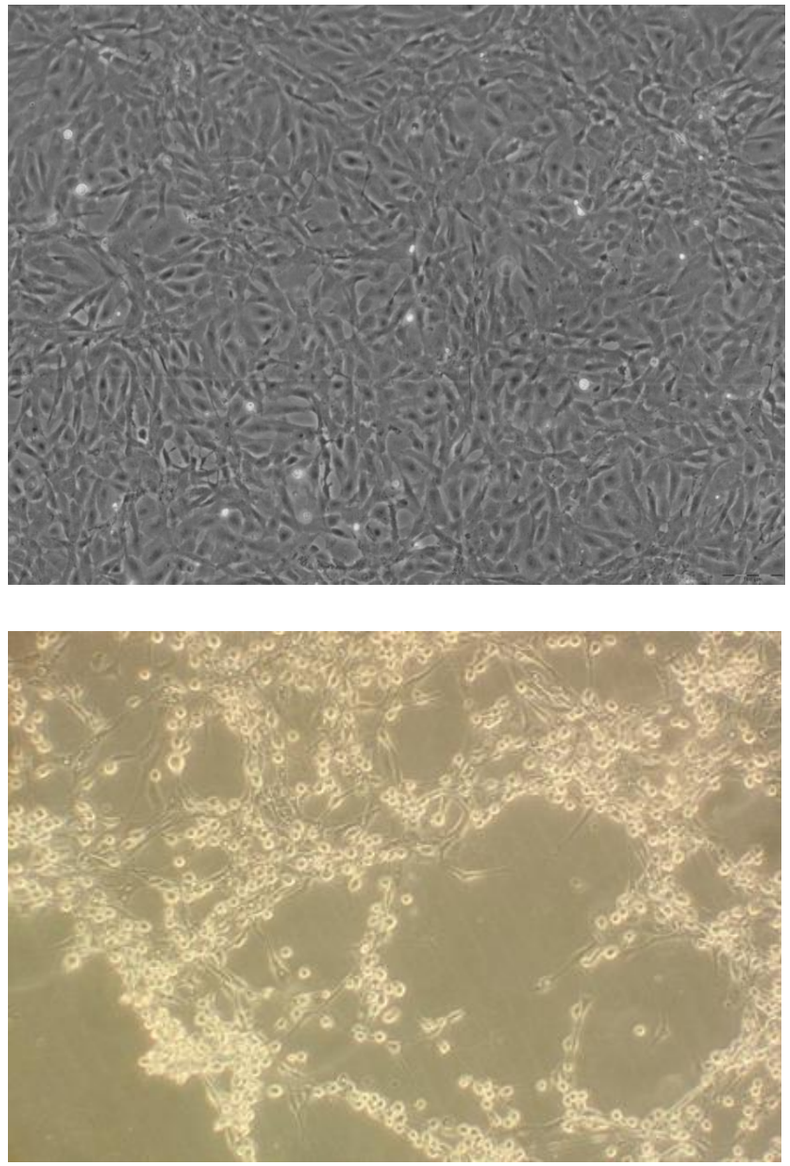
Establishment of Koi cell line

Koi-Fin



Cytopathic effect of Koi-Fin cells infected with KHV

Koi-Brain cells



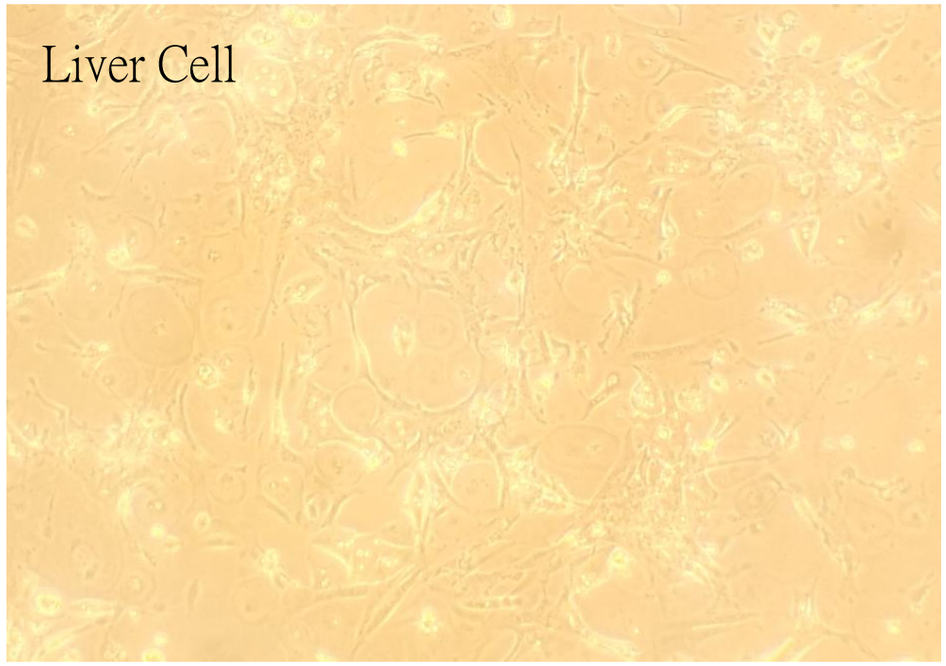
Cytopathic effect of Koi-Brain cells infected with KHV.

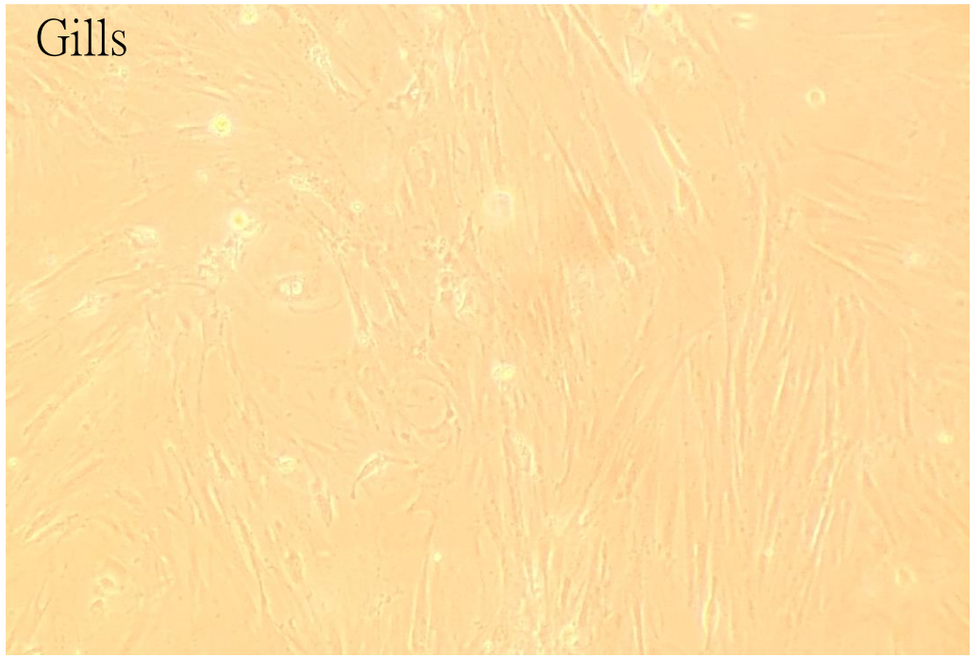
Virus titers of koi herpesvirus, on the course of koi brain cell passage up to passage level 25

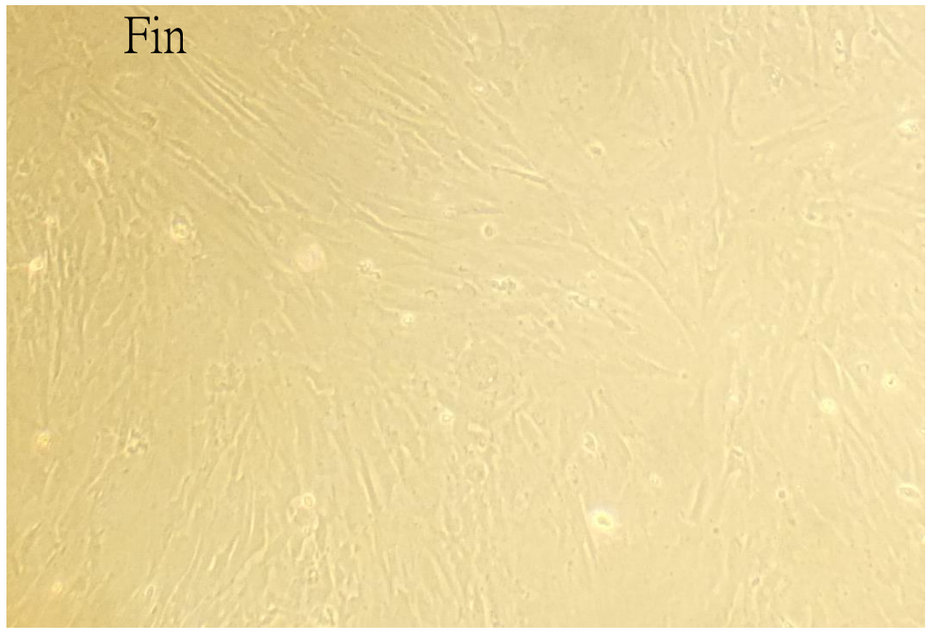
Viruses	TCID50	Realtime PCR/Ct	CPE
KHV-P1	10^5	14.53	5d 4+
KHV-P2	10^5	13.38	4d 4+
KHV-P3	10^5	13.15	4d 4+
KHV-P4	$10^{5.33}$	13.09	4d 4+
KHV-P5	10^6	11.51	3d 4+
KHV-P6	10^7	11.86	3d 4+
KHV-P7	$10^{7.33}$	11.31	3d 4+
KHV-P8	$10^{7.67}$	10.56	3d 4+
KHV-P9	$10^{7.5}$	11.71	3d 4+
KHV-P10	10^8	11.85	3d 4+
KHV-P11	$10^{7.67}$	11.78	3d 4+
KHV-P12	10^8	11.73	3d 4+
KHV-P13	$10^{7.67}$	11.83	3d 4+

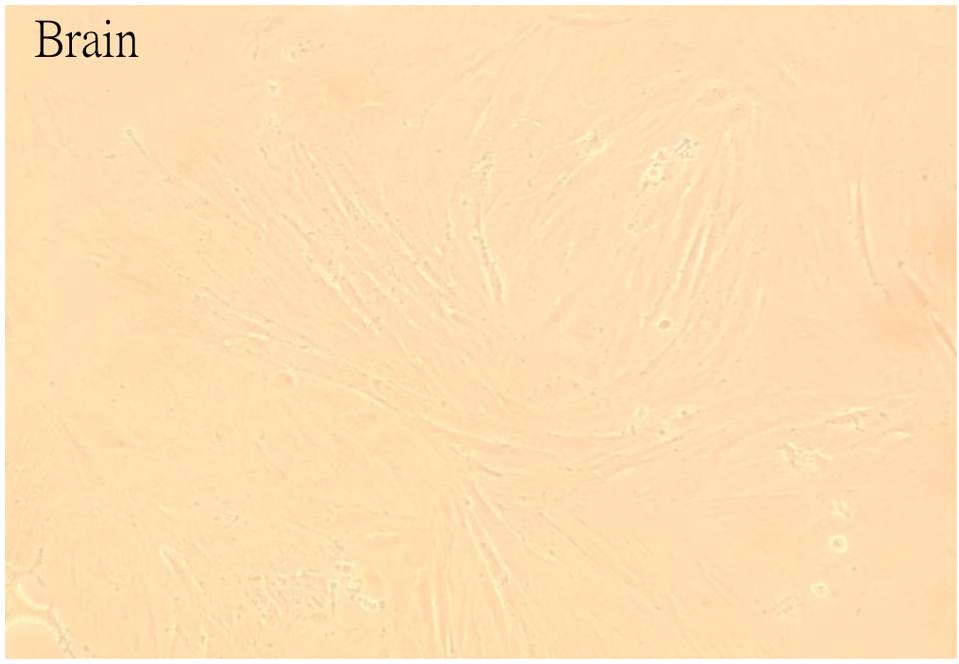
Viruses	TCID50	Realtime PCR/Ct	CPE
KHV-P14	$10^{7.67}$	11.27	3d 4+
KHV-P15	$10^{7.5}$	11.49	3d 4+
KHV-P16	10^8	10.1	3d 4+
KHV-P17	10^8	10.2	3d 4+
KHV-P18	$10^{7.67}$	11.04	3d 4+
KHV-P19	$10^{7.5}$	11.28	3d 4+
KHV-P20	$10^{7.67}$	10.83	3d 4+
KHV-P21	$10^{7.67}$	10.4	3d 4+
KHV-P22	$10^{7.67}$	10.82	3d 4+
KHV-P23	$10^{7.67}$	10.88	3d 4+
KHV-P24	10^8	10.2	3d 4+
KHV-P25	10^8	10.12	3d 4+

Establishment of Grouper cell line

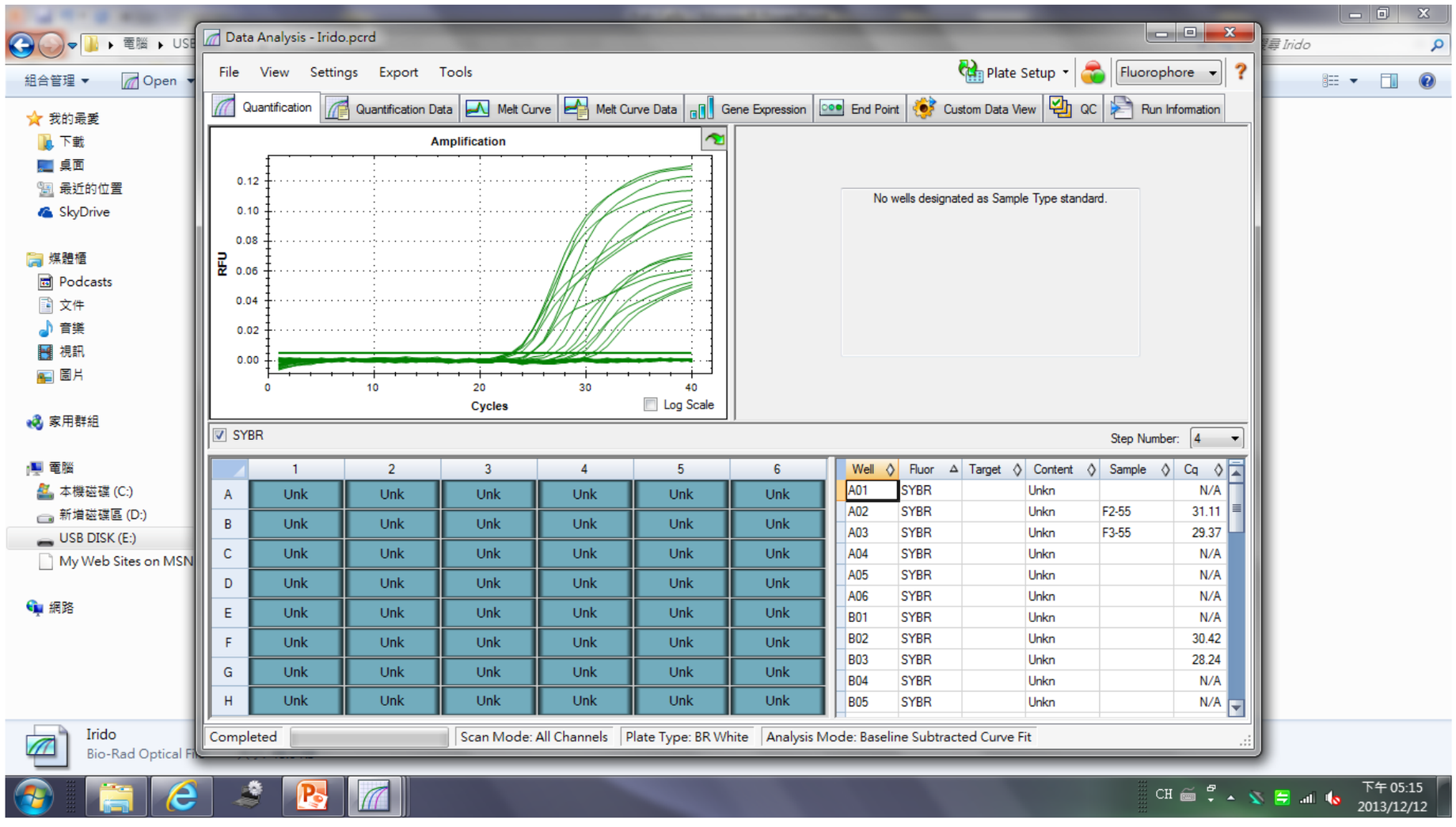








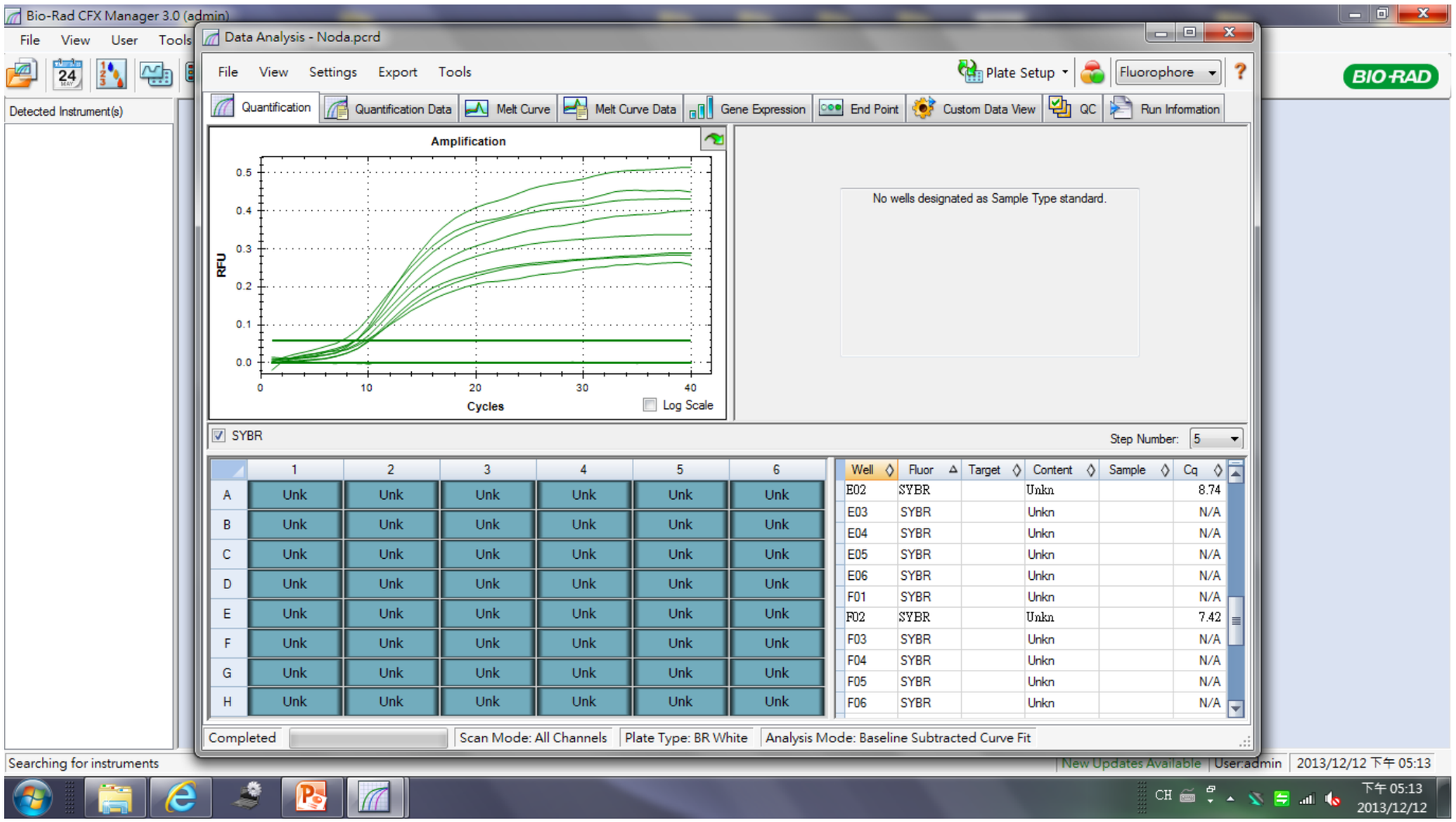
Iridovirus



	1	2	3	4	5	6
A	Unk	Unk	Unk	Unk	Unk	Unk
B	Unk	Unk	Unk	Unk	Unk	Unk
C	Unk	Unk	Unk	Unk	Unk	Unk
D	Unk	Unk	Unk	Unk	Unk	Unk
E	Unk	Unk	Unk	Unk	Unk	Unk
F	Unk	Unk	Unk	Unk	Unk	Unk
G	Unk	Unk	Unk	Unk	Unk	Unk
H	Unk	Unk	Unk	Unk	Unk	Unk

Well	Fluor	Target	Content	Sample	Cq
A01	SYBR		Unkn		N/A
A02	SYBR		Unkn	F2-55	31.11
A03	SYBR		Unkn	F3-55	29.37
A04	SYBR		Unkn		N/A
A05	SYBR		Unkn		N/A
A06	SYBR		Unkn		N/A
B01	SYBR		Unkn		N/A
B02	SYBR		Unkn		30.42
B03	SYBR		Unkn		28.24
B04	SYBR		Unkn		N/A
B05	SYBR		Unkn		N/A

Nodavirus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Quantification
Quantification Data
Melt Curve
Melt Curve Data
Gene Expression
End Point
Custom Data View
QC
Run Information
Amplification
RFU
Cycles
Log Scale
No wells designated as Sample Type standard.
SYBR
Step Number: 5
1 2 3 4 5 6
A Unk Unk Unk Unk Unk Unk
B Unk Unk Unk Unk Unk Unk
C Unk Unk Unk Unk Unk Unk
D Unk Unk Unk Unk Unk Unk
E Unk Unk Unk Unk Unk Unk
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G Unk Unk Unk Unk Unk Unk
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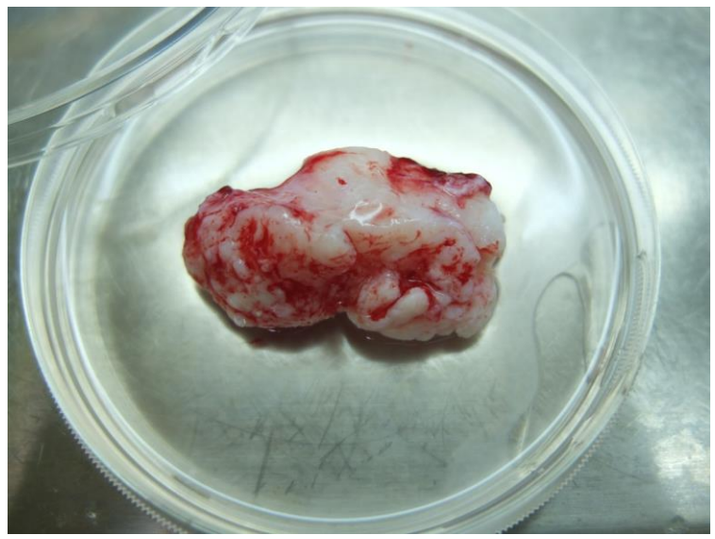
CH
下午 05:13
2013/12/12

Cell lines obtained from canine tumors

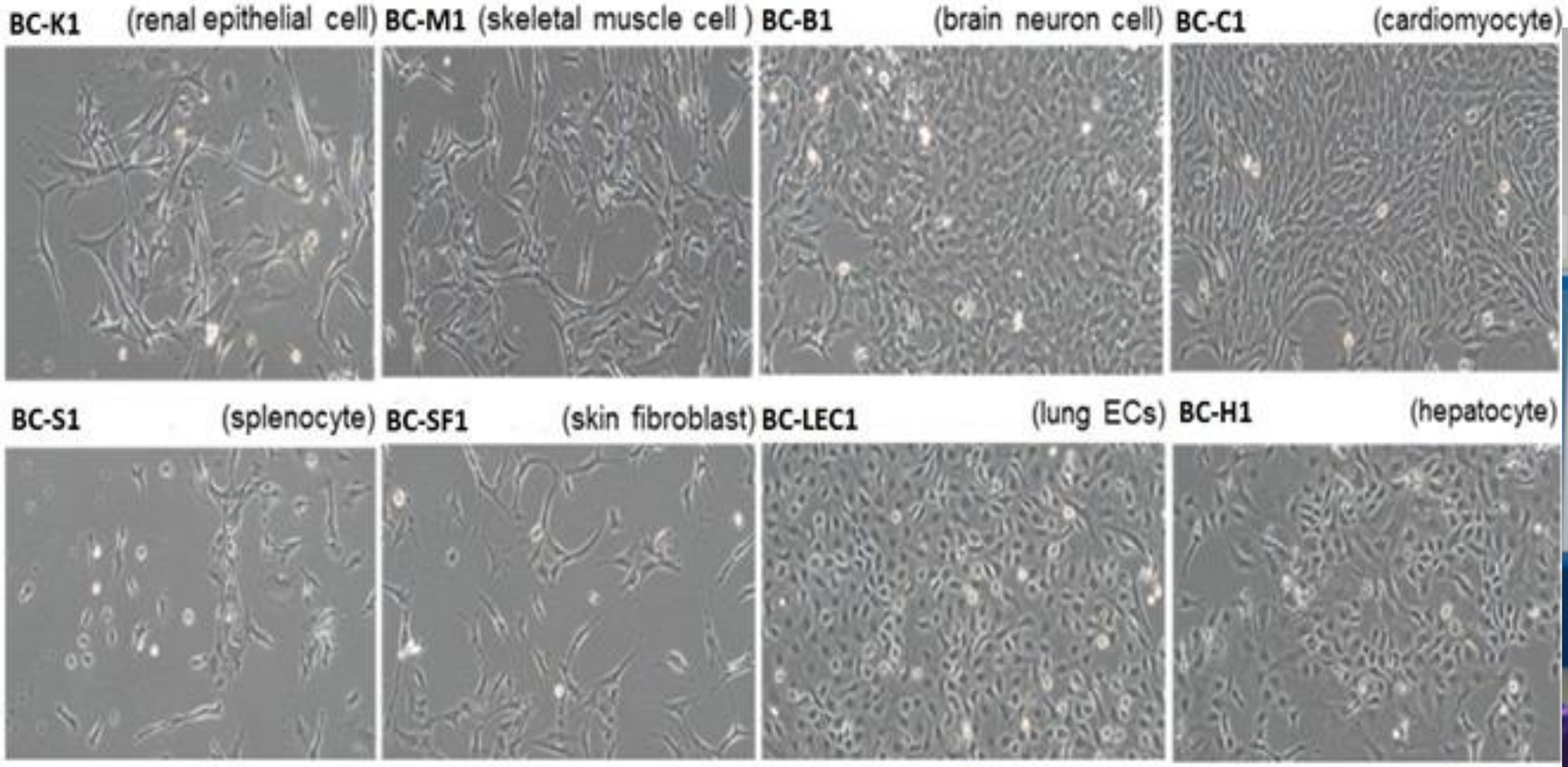
Adenomyoepithelioma

Lung tumor

Lipid stem cell



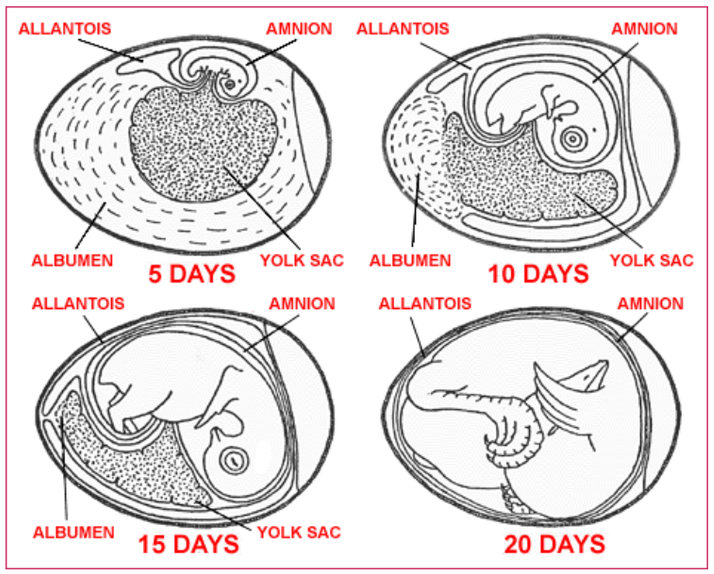
Bat Cell Line



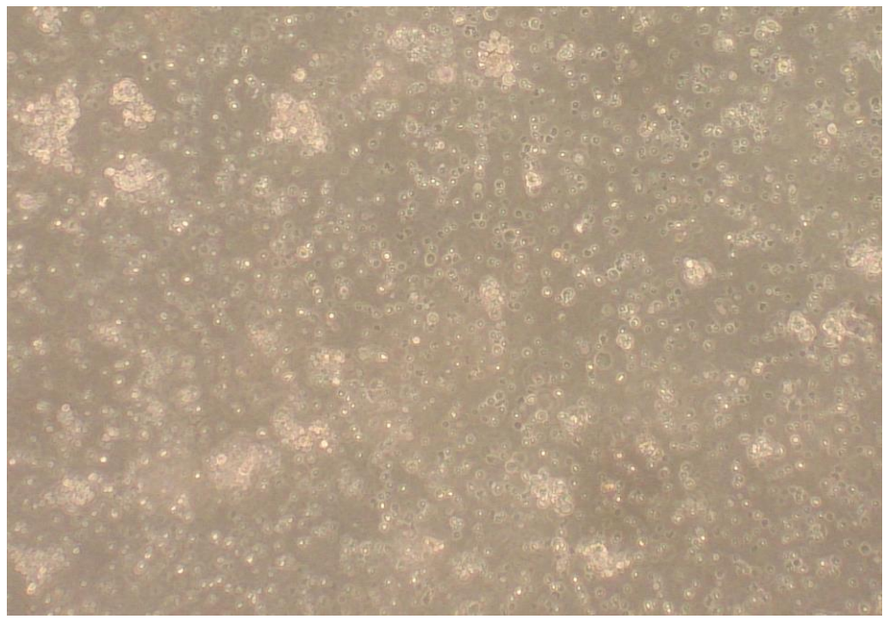
1. SARS-Cov2、MERS與PEDV等新興冠狀病毒冠狀病毒基因與蝙蝠冠狀病毒具有親源性，顯示蝙蝠可能是新興冠狀病毒的自然宿主，同時蝙蝠細胞具有運用於疫苗生產的潛力。

2.目前沒有商業化蝙蝠細胞株被開發出來。從高頭蝠(Scotophilus kuhlii)分離臟器與組織細胞，成功培養蝙蝠細胞株如下: (1) BC-B1 (腦神經細胞); (2) BC-LEC1 (肺臟內皮細胞); (3) BC-C1 (心肌細胞); (4) BC-H1 (肝臟細胞) (5) BC-S1 (脾臟細胞); (6) BC-K1 (腎小管上皮細胞); (7) BC-SF1 (皮膚纖維母細胞) ; (8) BC-M1 (骨骼肌細胞)。

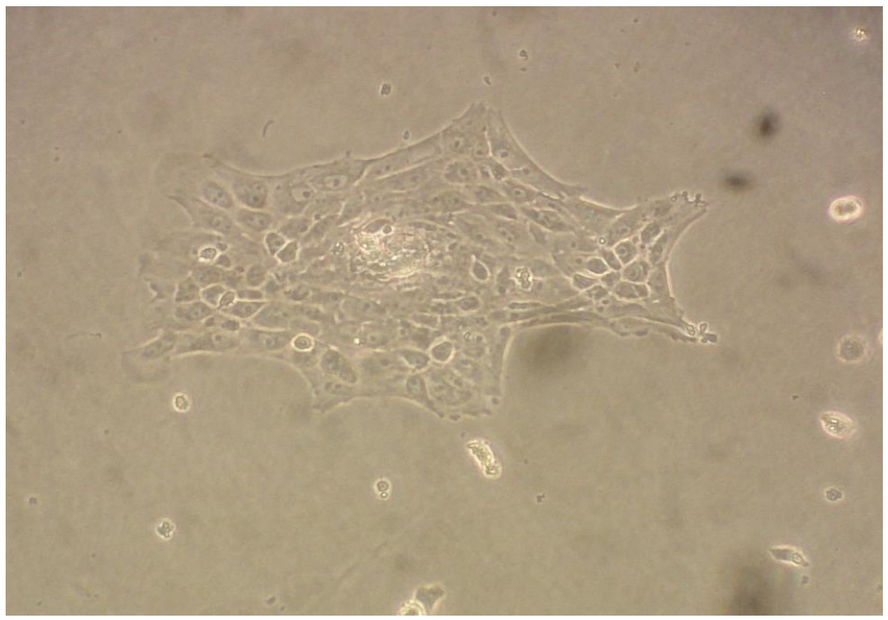
Chicken Embryo Cells



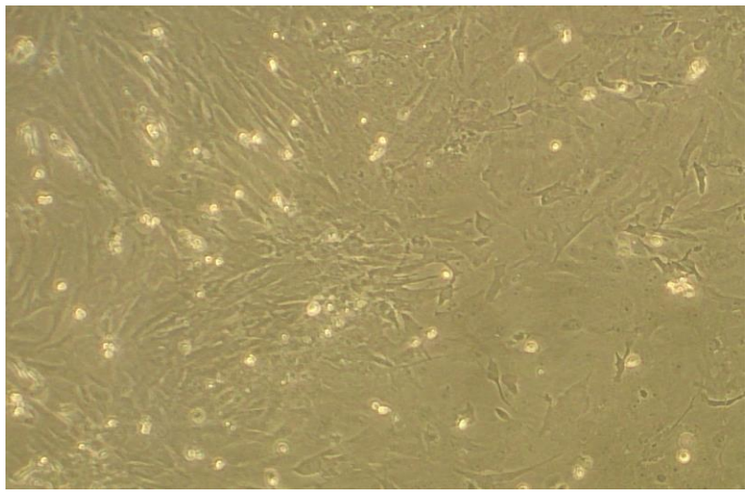
Bursa Cell



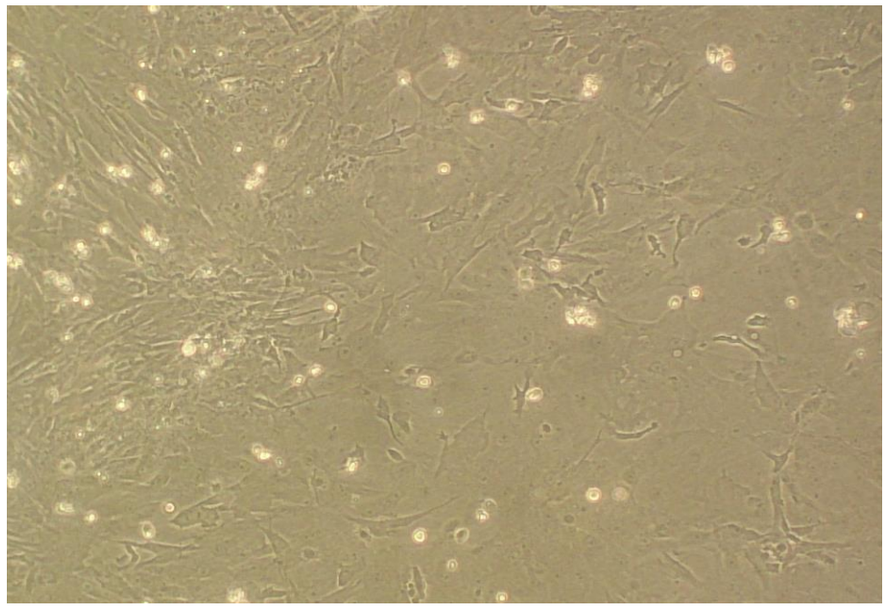
Brain Cell



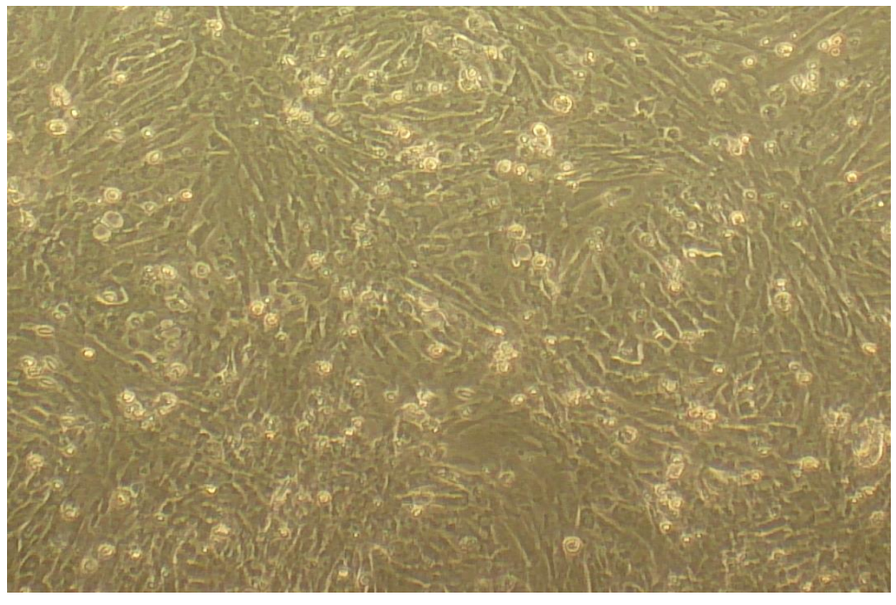
CEF



CAM



CEK

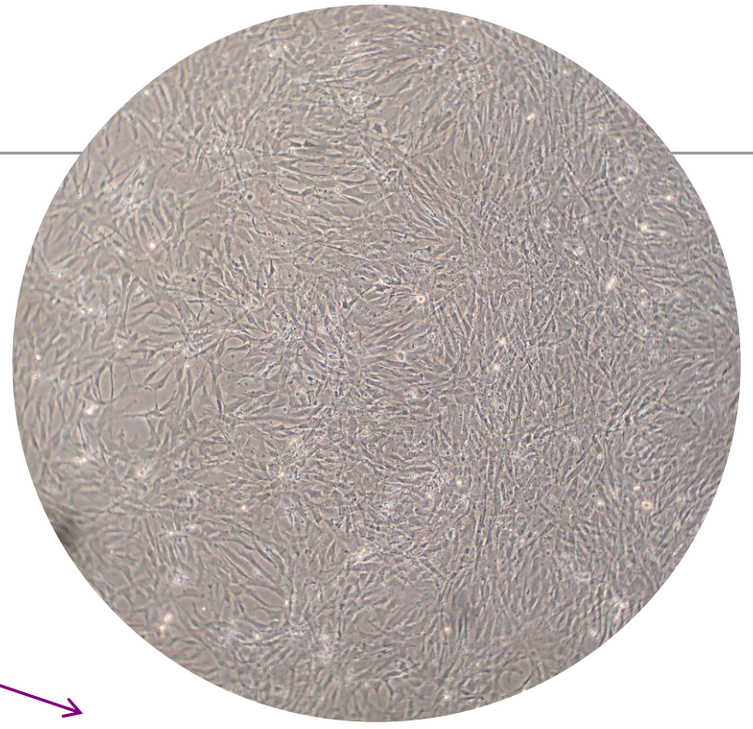


Primary cell —CEF(P1)

↓ 12-18 hrs

Cell attached and growth

1 hr



CEF/E6-P40
morphology

E6 vector transfection

E7 vector transfection

↓ 24 hrs

Cell Passage(P2)

↓ 4-5 days, G418

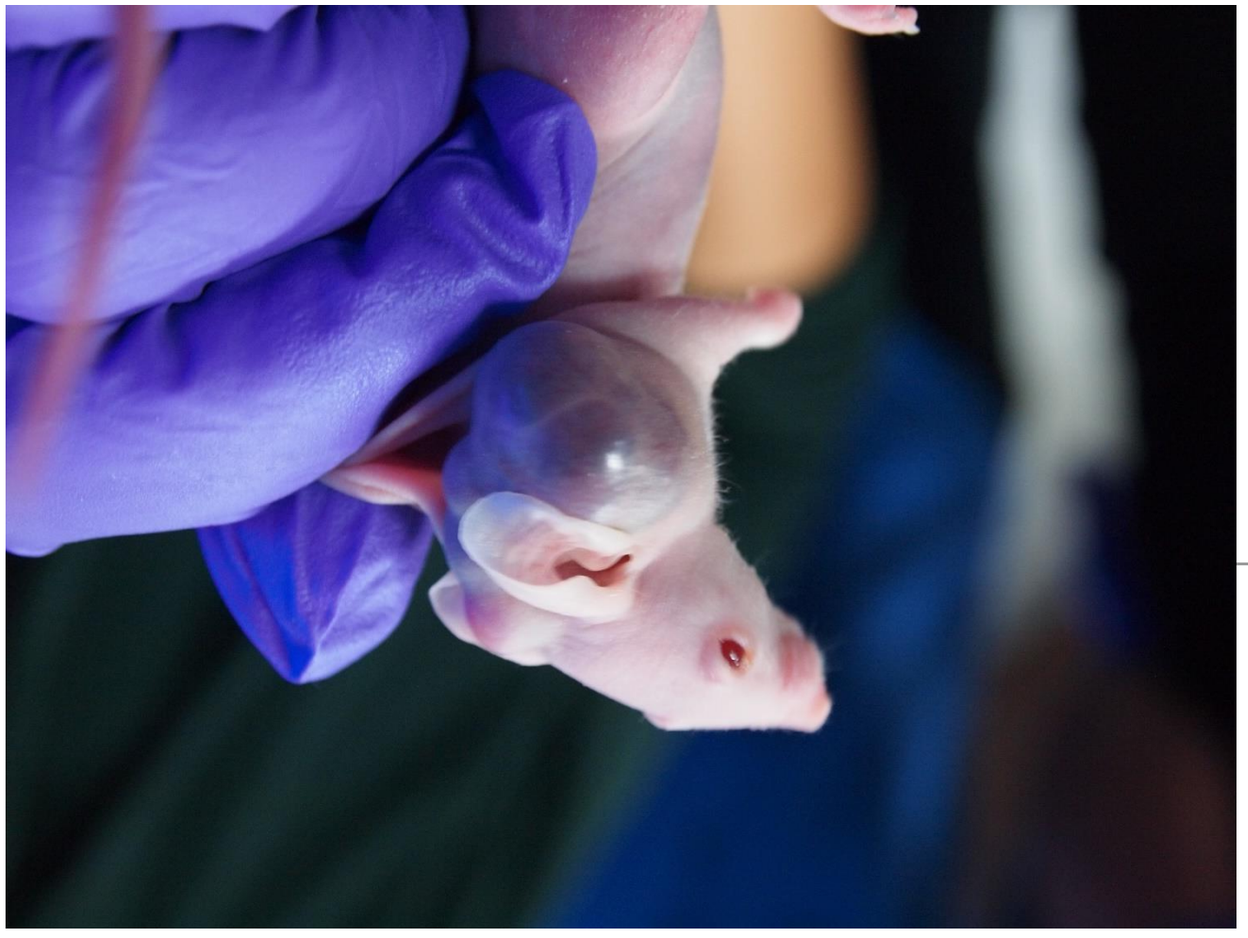
Cell Passage 細胞凍存

致瘤性試驗

- 用無胸腺小鼠至少10只，各皮下或肌肉注射$10^{7.0}$個被檢測細胞；同時用Hela或Hep-2細胞或其他適宜細胞系作為陽性對照細胞，每只小鼠各注射$10^{6.0}$個細胞；用二倍體細胞株或其他適宜細胞作為陰性對照細胞。

- 動物觀察14日，檢查有無腫瘤形成。如果有結節或可疑病灶，應繼續觀察至少1~2週，然後解剖，進行病理組織學檢查，應無腫瘤形成。對未發生結節的動物，取其半數，觀察21日，對另外半數動物觀察12週，對接種部位進行解剖和病理學檢查，觀察各淋巴結和各器官中有無結節形成，如果可疑，應進行病理組織學檢查，不應有轉移瘤形成。

- 陽性對照組觀察21日，應出現明顯的腫瘤。陰性對照組觀察21日，應為陰性。



生產用細胞標準

- **初代細胞**生產用禽源原代細胞應來自健康家禽的正常組織。每批細胞應按下列各項要求進行檢驗，任何一項不合格者，不得用於生產，已用於生產者，產品應予以銷毀。
  - **無菌檢驗**。
  - **黴漿菌檢驗**。
  - **外源病毒檢驗**。每批細胞至少取75cm²的單層，應無外源病毒汙染。

生產用細胞標準

- **細胞株**生產用細胞株一般由人或動物腫瘤組織或發生突變的正常細胞繼代而來；或者是通過選殖培養，從初代培養物或細胞株中獲得的具有特殊生長、生化性質或併敏感性的細胞群。
    - 應保存細胞株的完整記錄，如細胞來源、繼代歷史、培養基等。
    - 應對每批細胞的可見特徵進行監測，如細胞形態、生長速度、病毒感受性等。
- **無菌檢驗，黴漿菌檢驗，外源病毒檢驗**。

細胞庫建置和及管理

Primary Cell Bank，PCB：由一個原始細胞群體發展成為穩定繼代的細胞群體，或經過選殖培養形成的均一細胞群體，經無菌試驗、純潔試驗等檢驗後於液氮凍存，即為原始細胞庫，供建立主細胞庫用。

Master Cell Bank，MCB ：原始細胞庫細胞傳代增殖後均勻混合成一批，定量分裝，保存於液氮。這些細胞須按其特定工作需求進行全面檢定(病毒感受性、增值速率……)，全部合格後即為MCB，供建立工作細胞庫用。主細胞庫的質量標準應高於初級細胞庫。生產企業的MCB至多不得超過兩個細胞代次。

Working Cell Bank，WCB：經主細胞庫細胞繼代增殖，，定量分裝於一定數量的細胞凍存管，保存於液氮以下備用，即為工作細胞庫。凍存時細胞復甦後繼代增殖的細胞數量應能滿足生產一批生產。工作細胞庫的質量標準應在主細胞庫的質量標準基礎上建立。生產企業的工作細胞庫必須限定為一個細胞代次。

細胞庫管理

(1)每種細胞庫均應分別記錄放置位置、保存數量和取用記錄，確保儲存細胞在儲存期間能被準確找到。

(2)凍存的細胞存活率須在90%以上(特殊細胞視具體情況而定)。凍存後的細胞，應至少做一次解凍培養並連續繼代至衰老期，檢查不同代次的細胞生長情況。

(3)主細胞庫和工作細胞庫貯存條件應當一致，檢定和生產用各級細胞庫應分不同液氮桶存放，異地存放以降低細胞庫風險。非生產用細胞應與生產用細胞嚴格分開存放。

(4)細胞應當在適當溫度下保存，並有明確標籤，冷藏庫應有連續溫度記錄，應定期檢查液氮桶內液氮量並及時補加，液氮保藏環境溫濕度應做好記錄，任何偏離貯存條件的情況及糾正措施都應記錄。

(5)在指定人員的監督下，經批准的人員方可進行細胞庫操作，未經批准不得使用細胞庫。

(6)貯存期間的主細胞庫和工作細胞庫中的細胞一旦取出，不得再返回庫內貯存。

細胞	來源	穩定繼代代次	感受性病毒	備註
PK207	SPF Swine Kidney	>100 P	HCV, PCV2, PRRSV, PRV, TGEV, PEDV, Rotavirus	Spontaneous mutations
PS207	SPF Swine Spleen	>50 P	HCV, PCV2, PRRSV, PRV, TGEV, PEDV, Rotavirus	Spontaneous mutations
ST207	SPF Swine Testicles	>50 P	HCV, PCV2, PRRSV, PRV, TGEV, PEDV, Rotavirus	Spontaneous mutations
QEF	Quail embryo	>50 P	NDV, IBV, POXV, ARV, EDSV, PiCV	Spontaneous mutations
CA207	Canine Adenomyoepithelioma	>50 P	CDV, CPV	Cancer
CL207	Canine lung cancer	>50 P	CDV, CPV	Cancer
GL207	Grouper liver cell	>20 P	Iridovirus, NNV	Spontaneous mutations
GG207	Grouper gills cell	>20 P	Iridovirus, NNV	Spontaneous mutations
GS207	Grouper spleen cell	>50 P	Iridovirus, NNV	Spontaneous mutations
GF207	Grouper fin cell	>50 P	Iridovirus, NNV	Spontaneous mutations
GK207	Grouper kidney cell	>50 P	Iridovirus, NNV	Spontaneous mutations
EE-1	Eel endothelial cells	>50 P	Japanese eel endothelial cells-infecting virus	Spontaneous mutations
MK207	Monkey kidney	>50 P	Influenza A, Influenza B	Spontaneous mutations
KB207	Koi brain cell	>50 P	Koi Herpesvirus	Spontaneous mutations
KF207	Koi fin cell	>50 P	Koi Herpesvirus	Spontaneous mutations
CAS	Canine adipose stem cells	>50 P	CDV, CPV	Spontaneous mutations
FT-1	Feline Testicles	>50 P	FIP, CPV, FIV, FHSV, FCV	Spontaneous mutations
FT-2	Feline Testicles	>50 P	FIP, CPV, FIV, FHSV, FCV	Spontaneous mutations
FT-3	Feline Testicles	>50 P	FIP, CPV, FIV, FHSV, FCV	Spontaneous mutations

5

Serum-free suspension cell culture

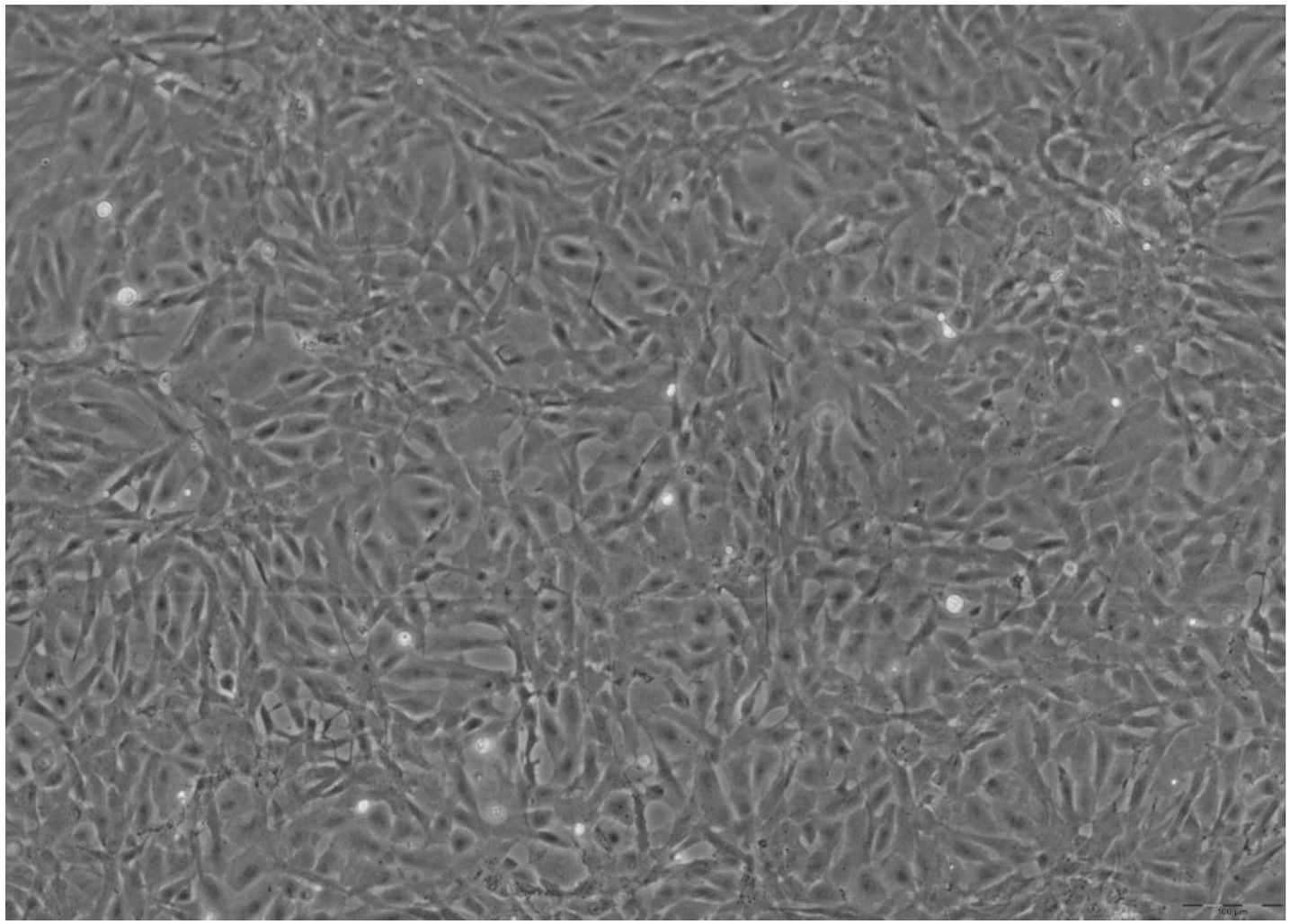
Principles

- For conventional industrial scale cell culture, the expensive components are:
  - Serum
  - Carrier

We adapt the cells to a serum-free environment and grow them in suspension.

抗原工業化量產

- 生物反應槽技術
- 種毒株篩選技術
- 細胞培養技術
- 培養基技術



- 大量培養 VS 高密度培養

生物反應槽/細胞生長載體

- 運用之產業

  -蛋白質藥物(第VII凝血因子)、標靶藥物.....

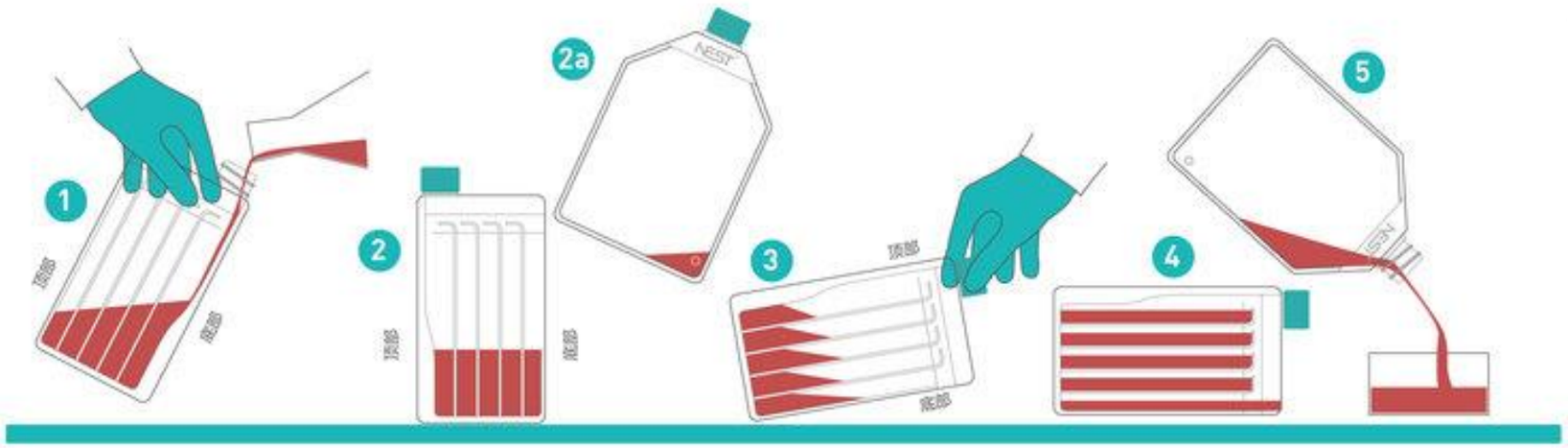
  -生技產業

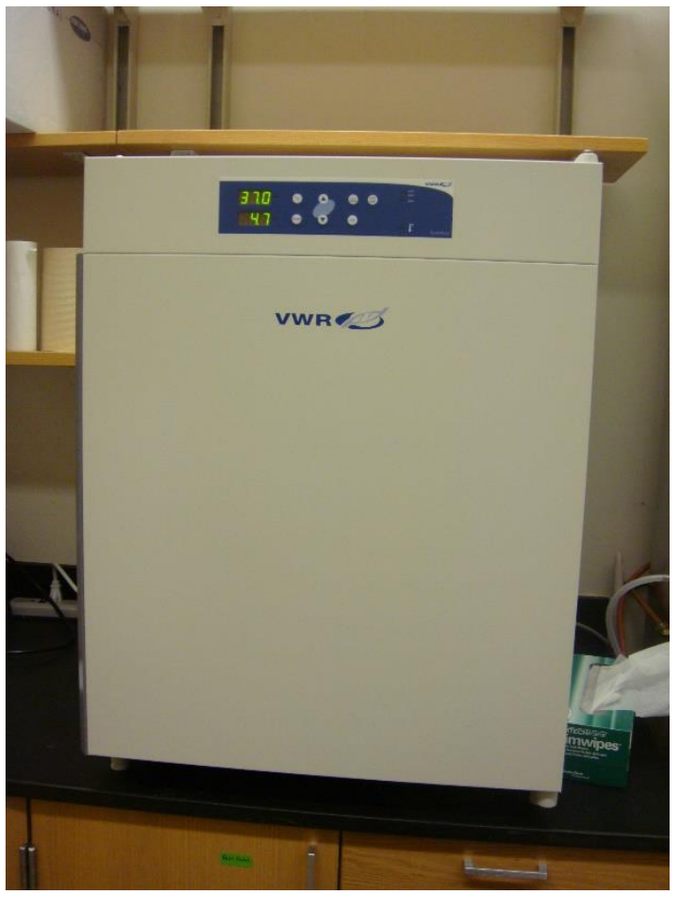
  -人用疫苗產業

  -動物用疫苗產業

- 生物反應槽單價：100萬至數億元
- 細胞生長載體單價：5-10萬/公斤

傳統細胞培養方法

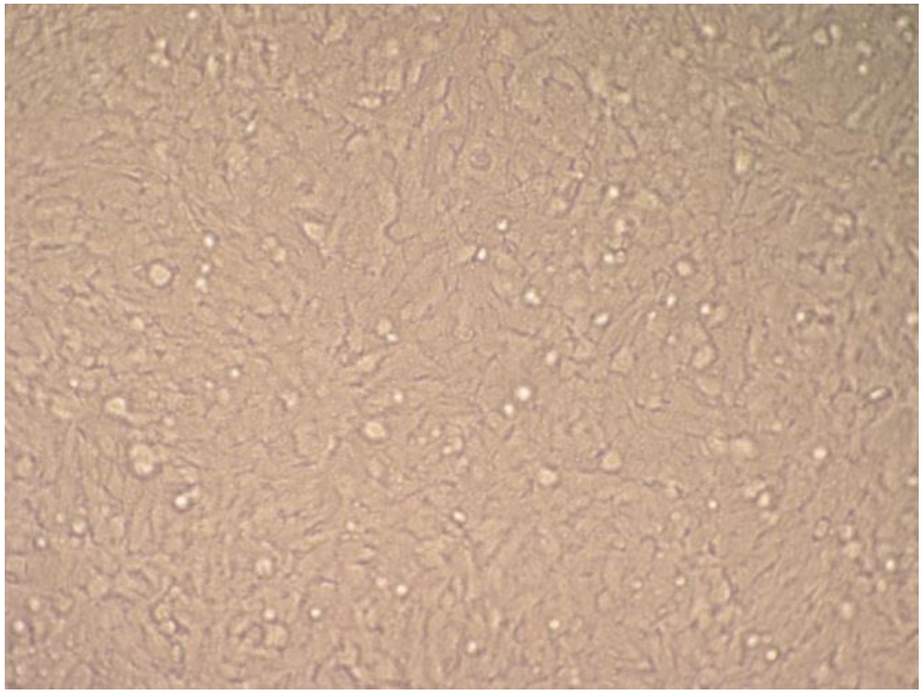




滾瓶培養

- 優點：固定投資少，簡單，技術成熟；

- 缺點：占地面積大，規模小，人力成本高，血清成本高，暴露環節多，產品品質不穩定。



生物反應槽培養優點

微載體培養

- 適用於目前各種主流的細胞培養和疫苗生產
- 用途廣，從角瓶和滾瓶培養可以直接轉換
- 便於饋料，取樣，連續培養

全懸浮培養

優點：培養簡單，長期成本低，易於觀察，放大容易規模大，操作環節少，自動化程度高。

缺點：前期技術要求高，技術開發時間長。

細胞培養生物反應器7L～500L

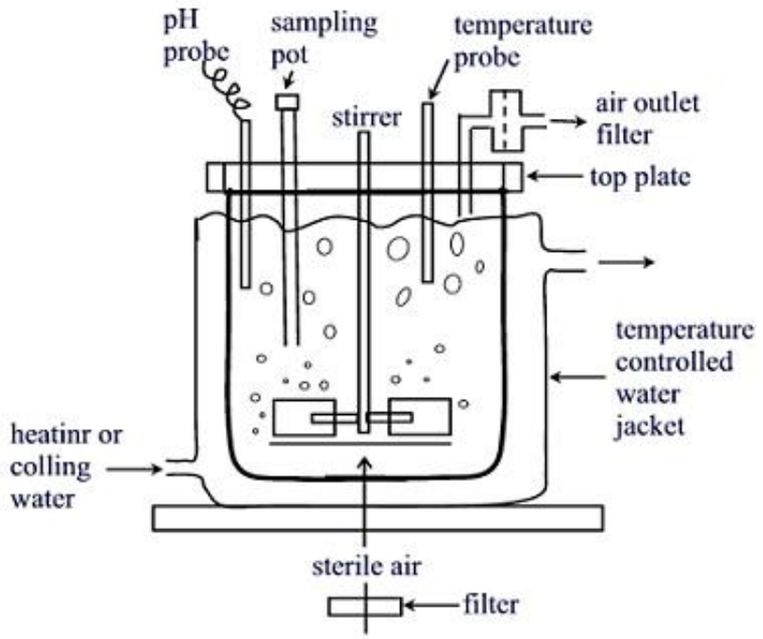
3L-7L-10L-14L

50L-75L-100L-150L-200L

300L-500L-1000L

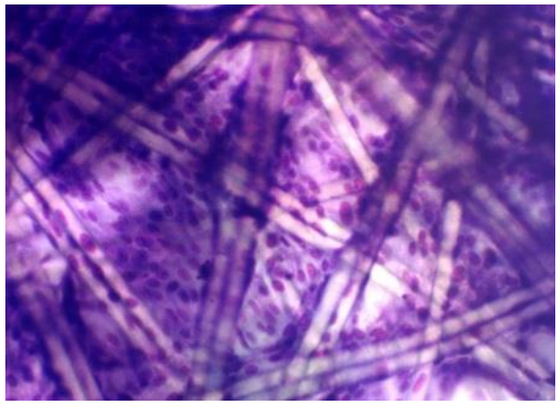
生物反應槽


RP GMP BREEDER
RP CONTROL UNIT

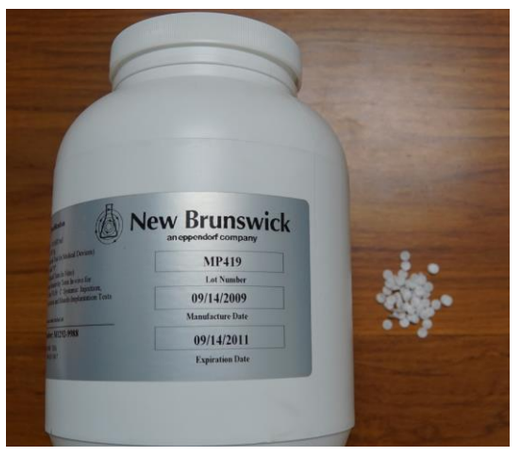

pH probe
sampling pot
temperature probe
stirrer
air outlet filter
top plate
temperature controlled water jacket
heatinr or colling water
sterile air
filter

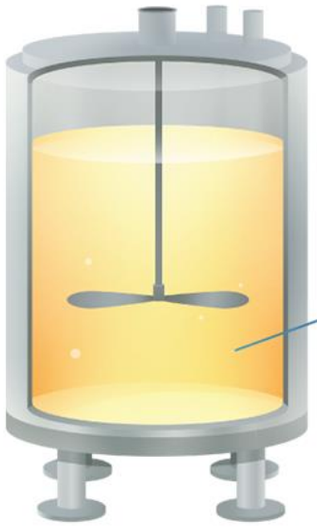

PALL

病毒大量培養設備

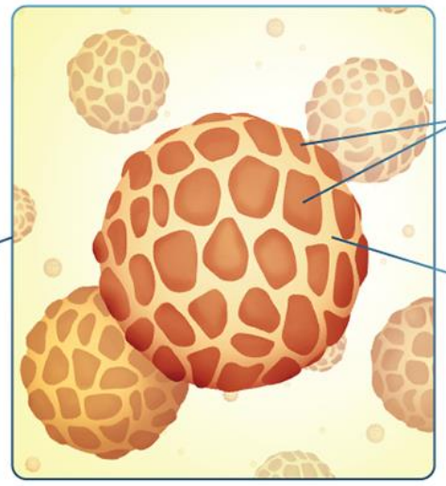


細胞生長載體


New Brunswick
an eppendorf company
MP419
Lot Number
09/14/2009
Manufacture Date
09/14/2011
Expiration Date



Microcarrier suspension



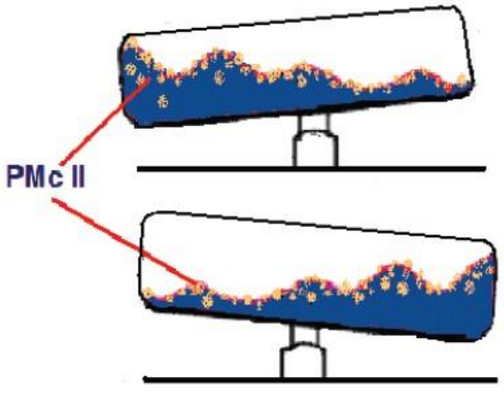
Cells grown on microcarrier

Density > Culture media

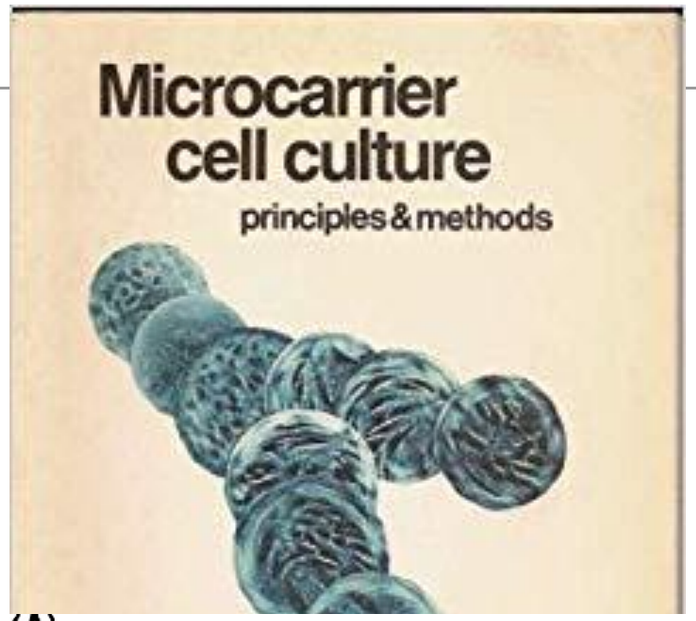
Sensitive to Mc Conc.

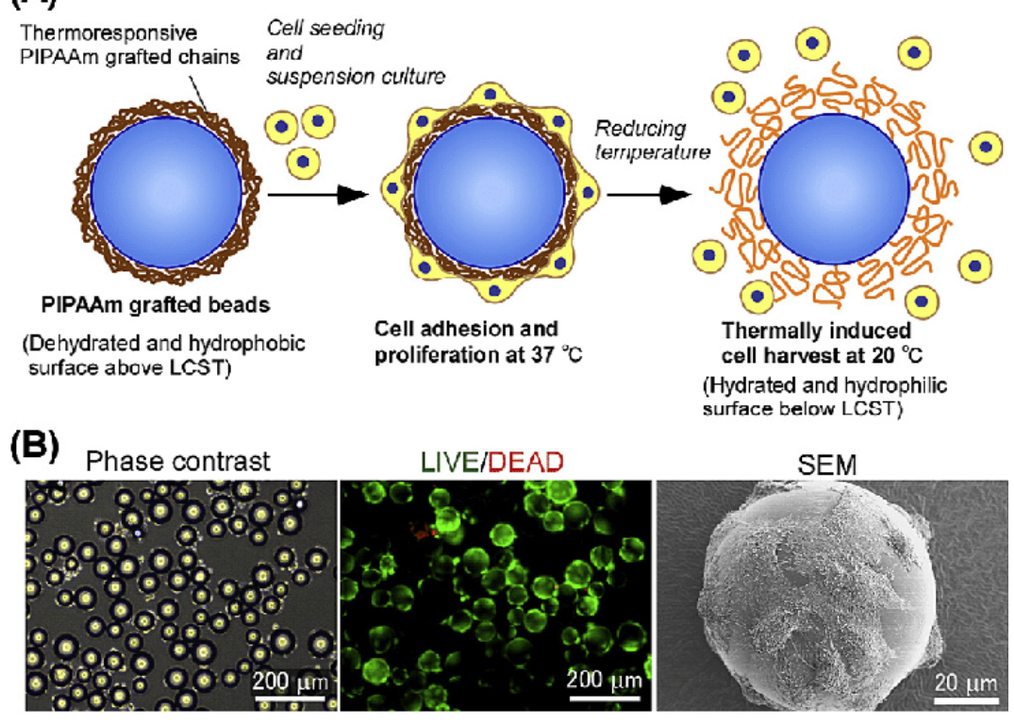
Agitation & O_2

Density ≤ Culture media



Little or no Limitations for High Density Culture



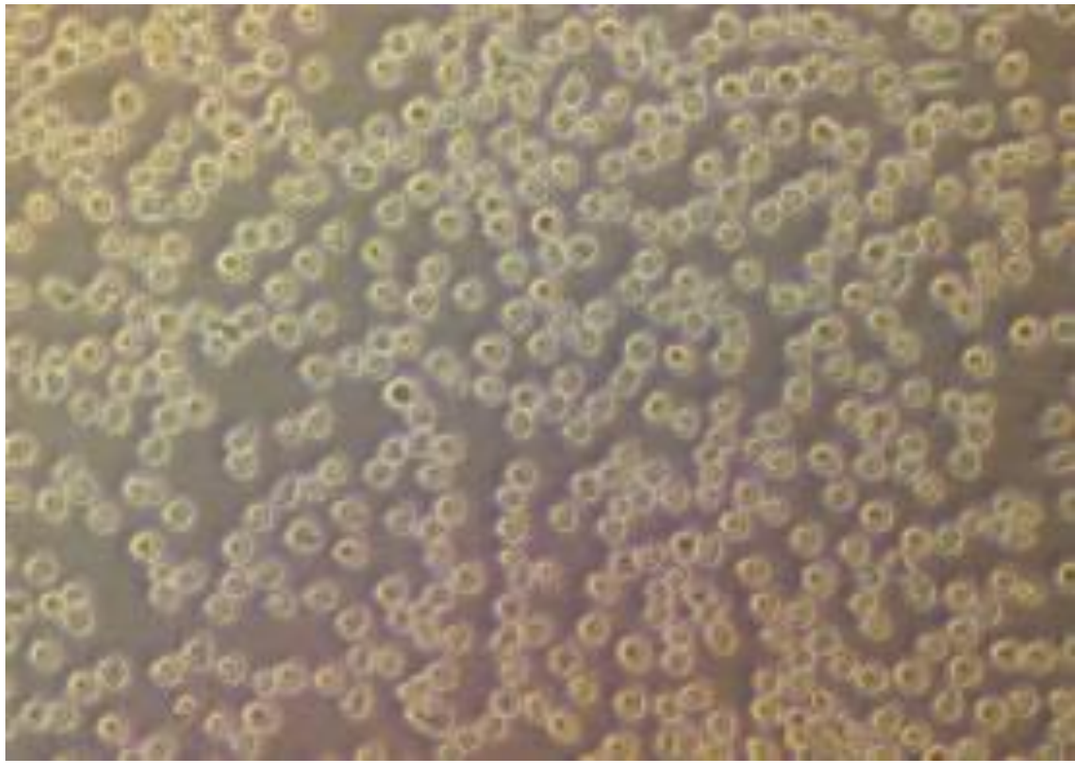


懸浮培養

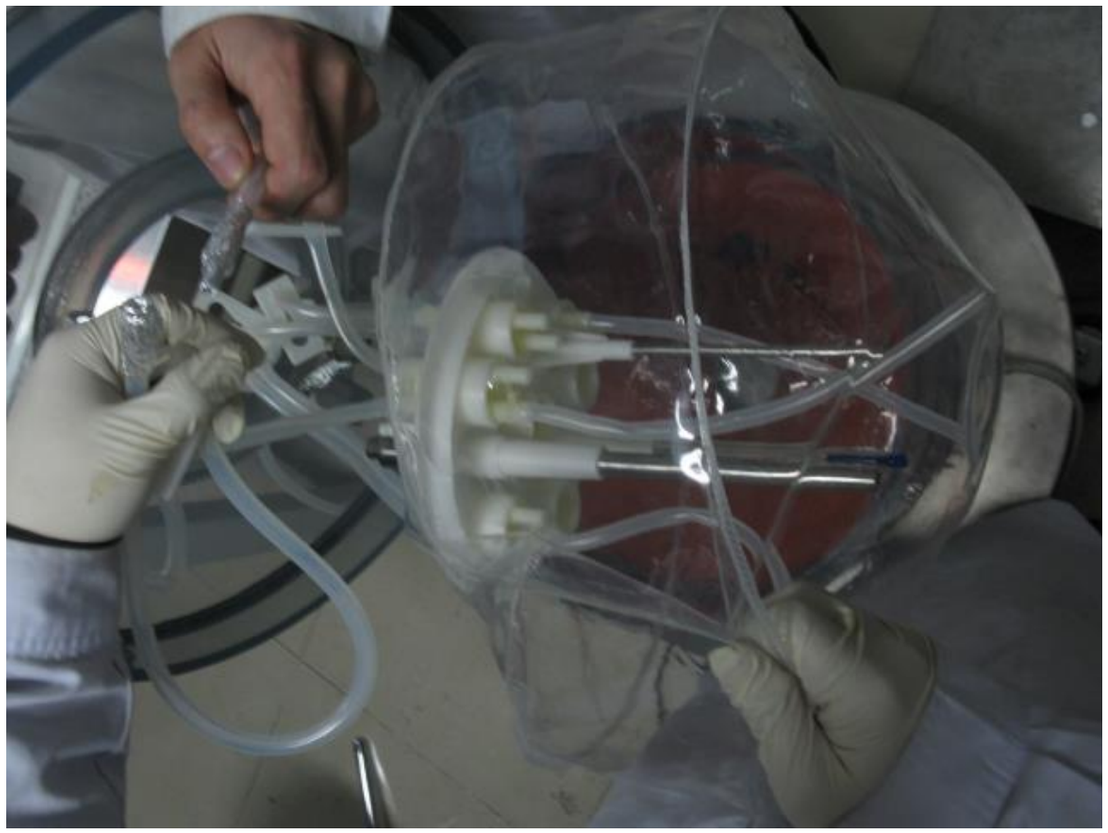
適用細胞株：融合瘤、CHO、BHK、MDCK、昆蟲細胞等　。

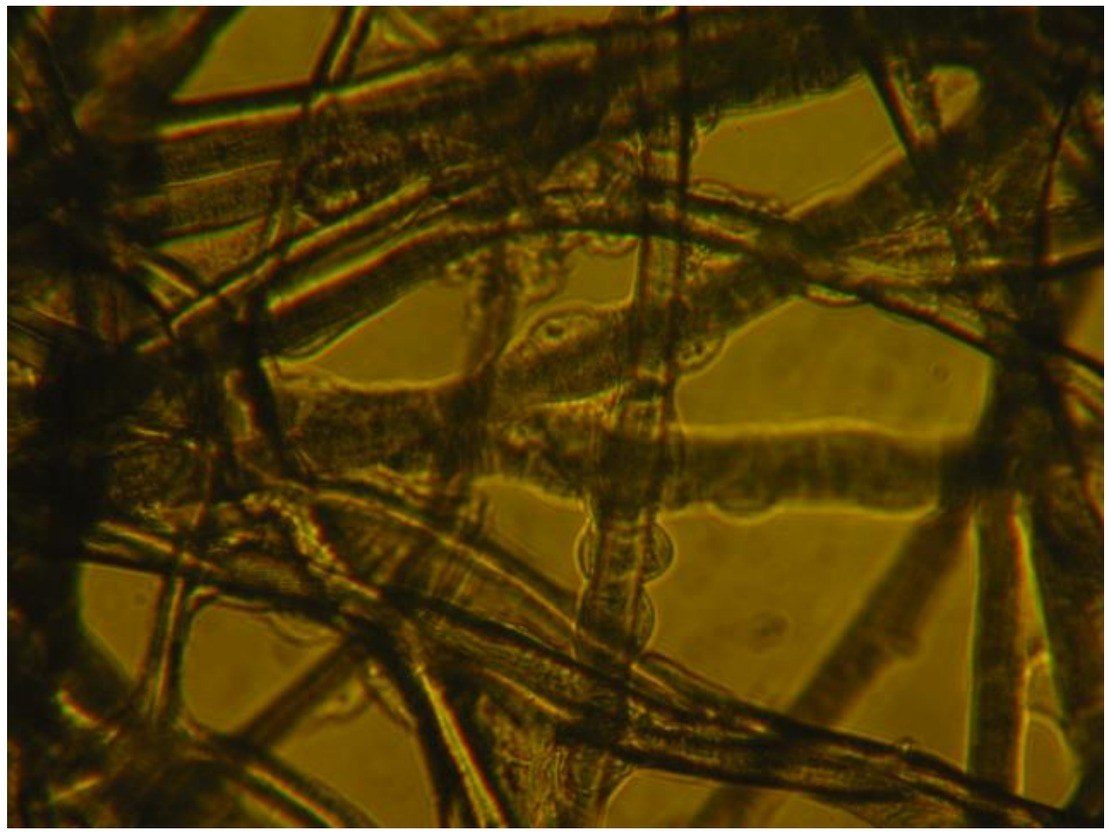
優點：操作簡便，易放大，成本低。

局限性：馴化難，病毒適應性，使用範圍受限。

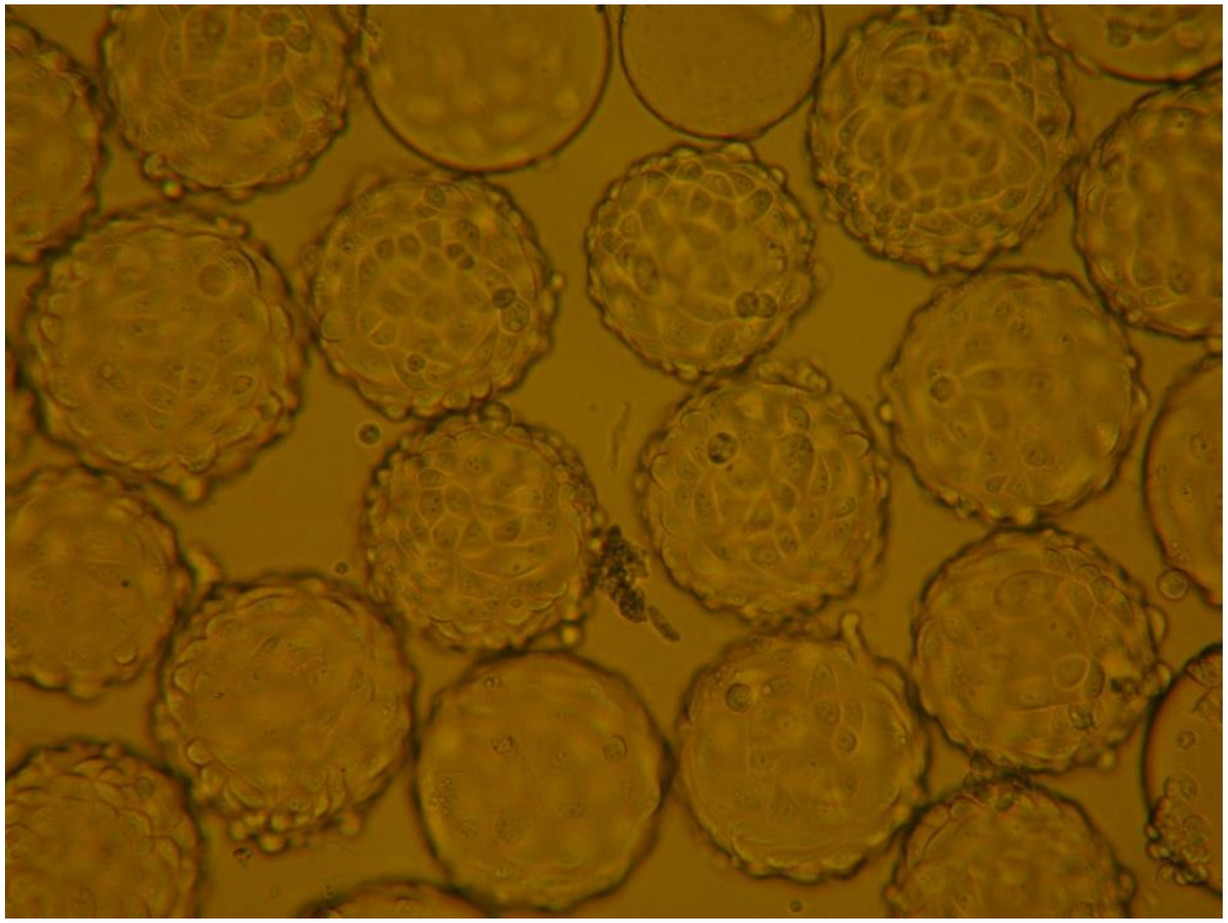


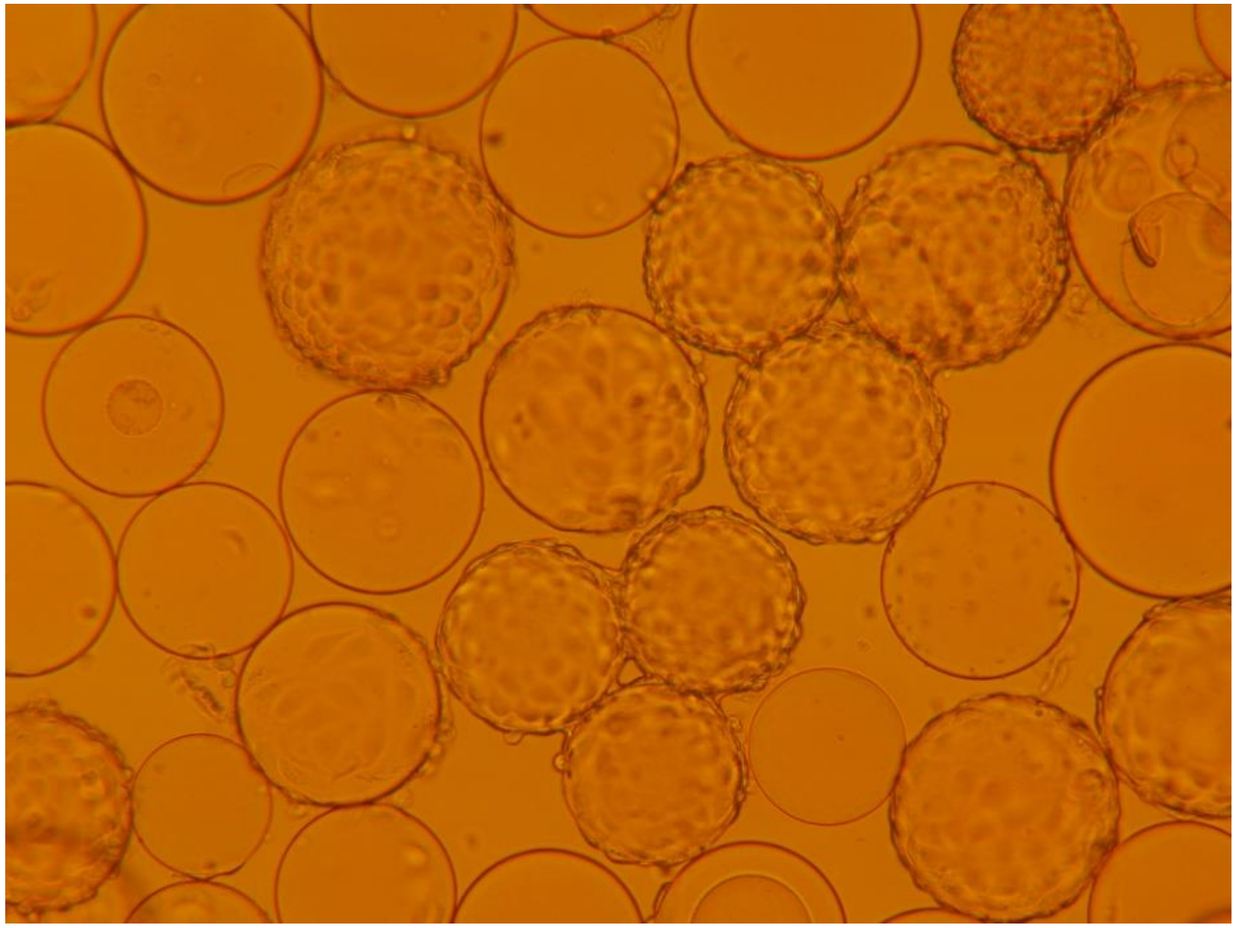
纖維載體



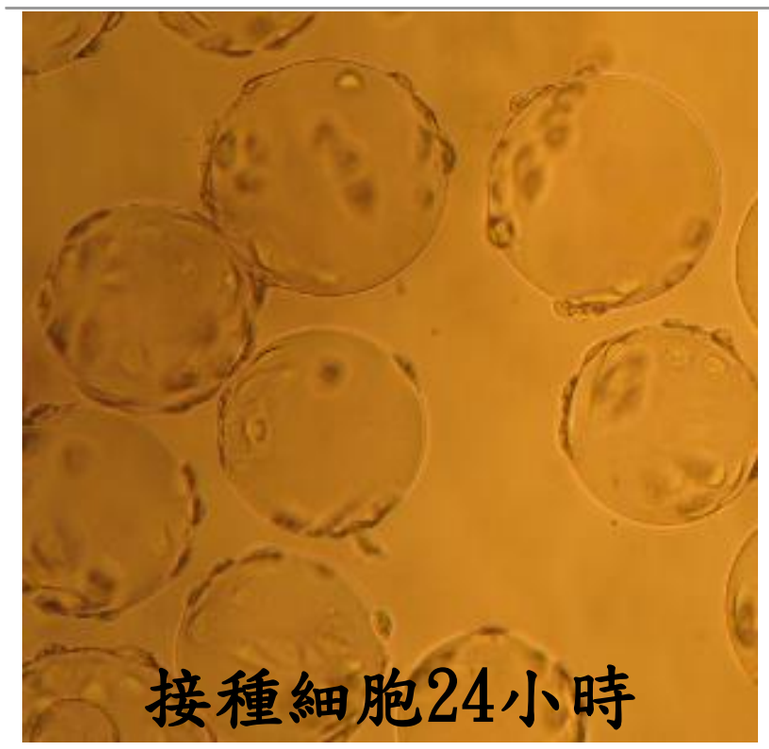


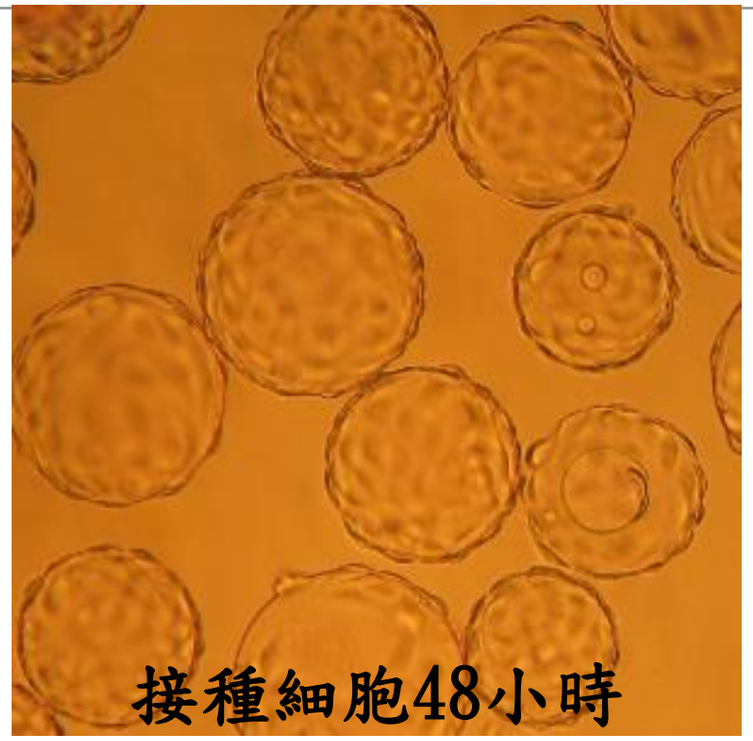
球狀微載體

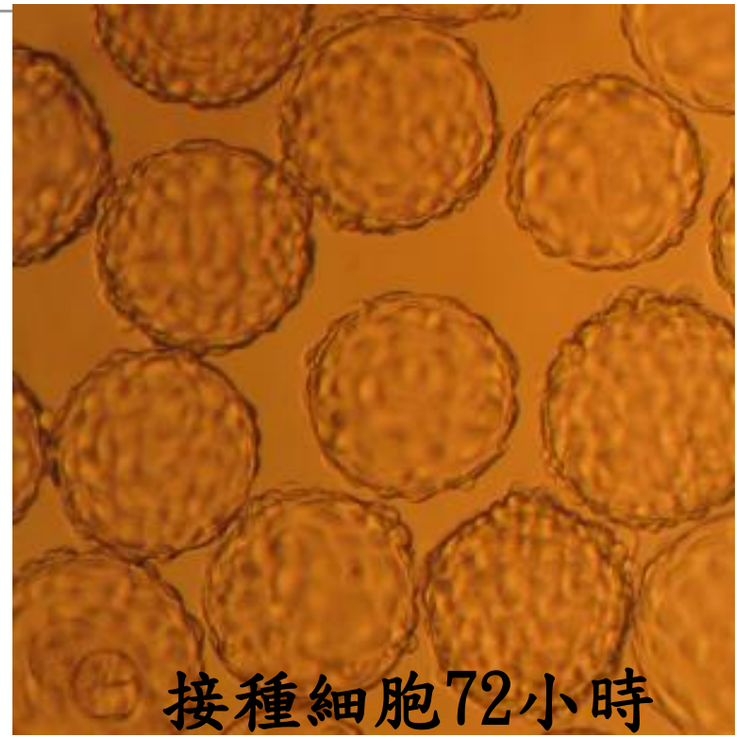


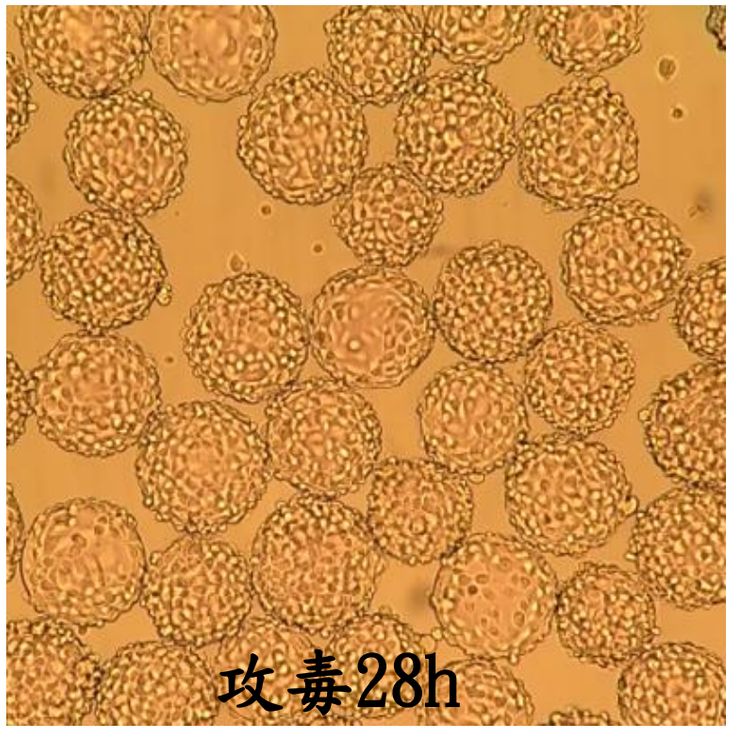


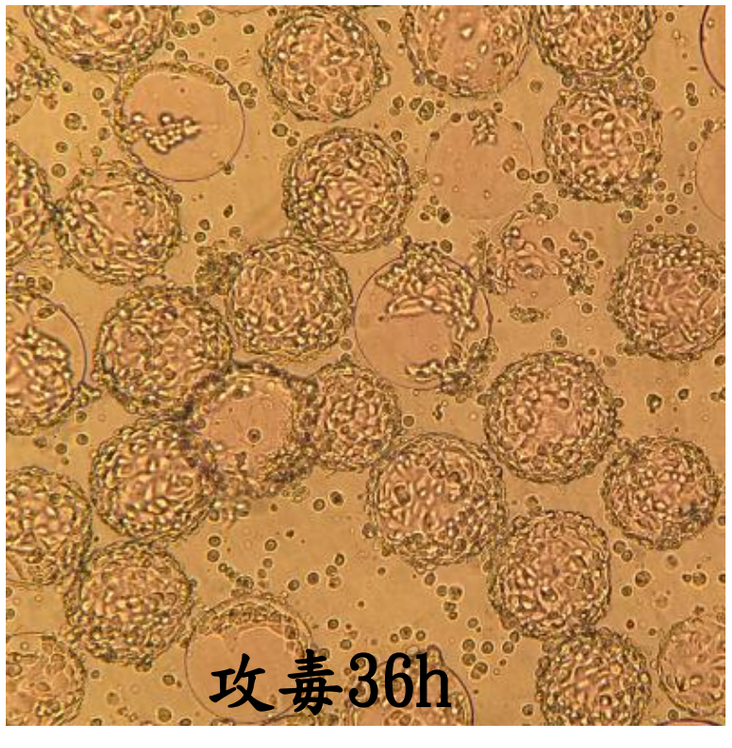
Marc-145/ PRRSV

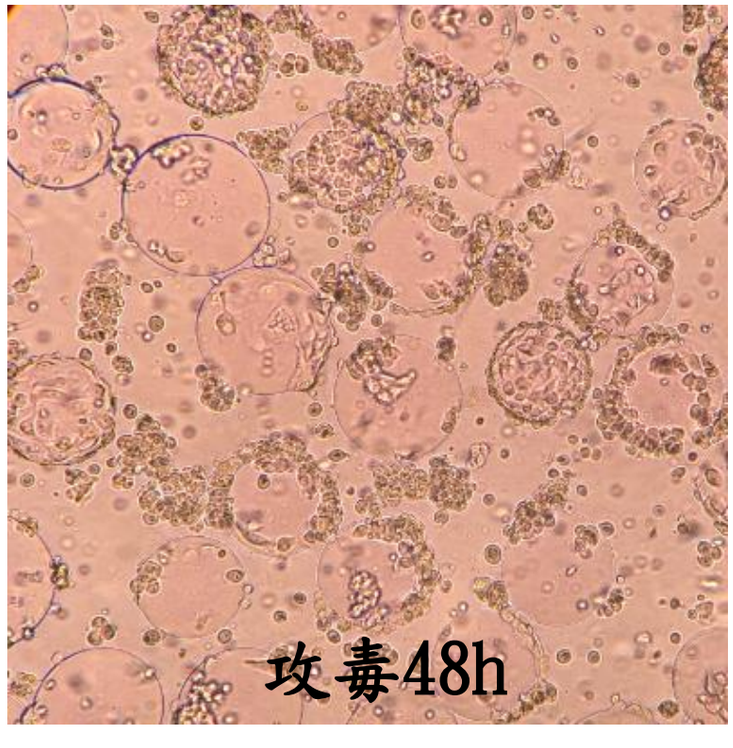


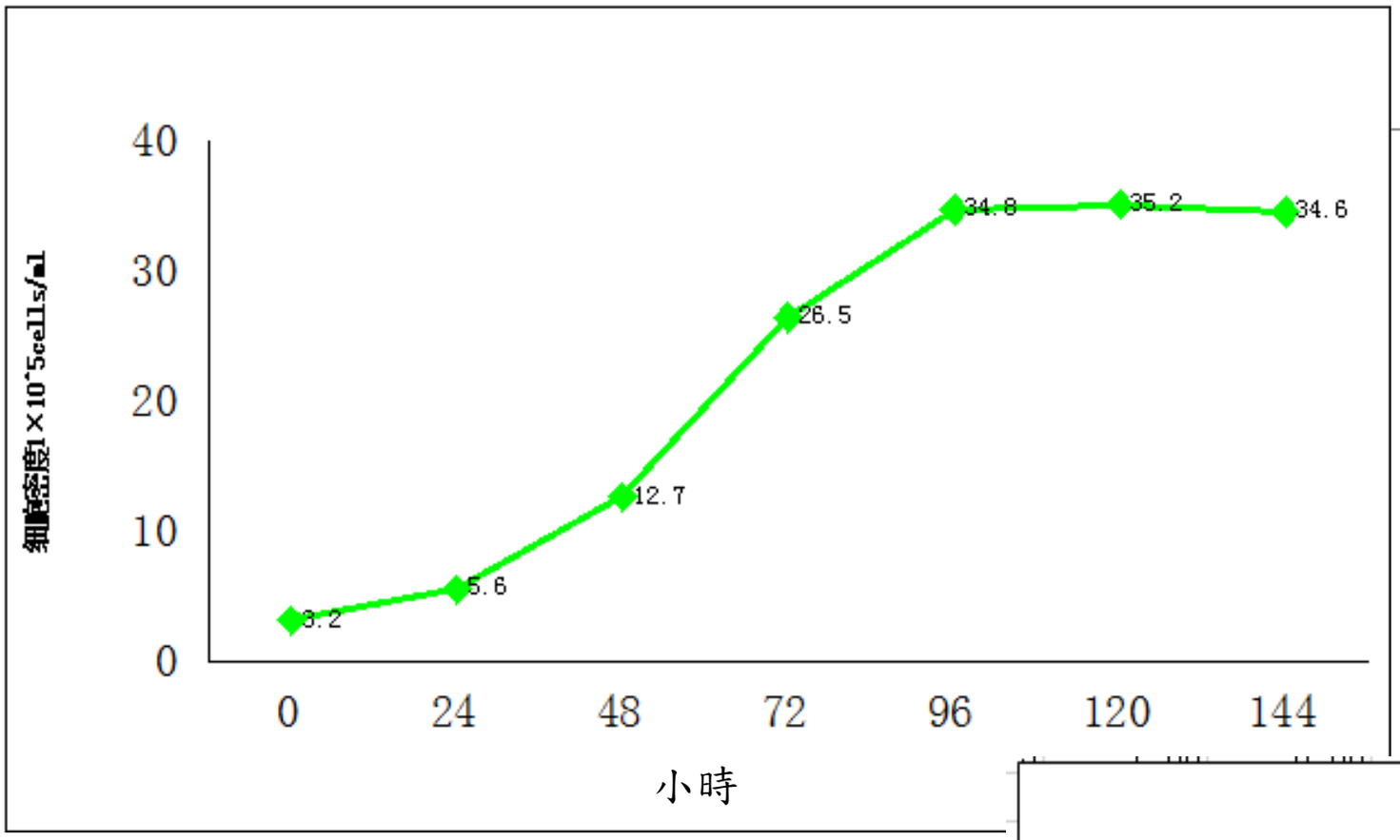




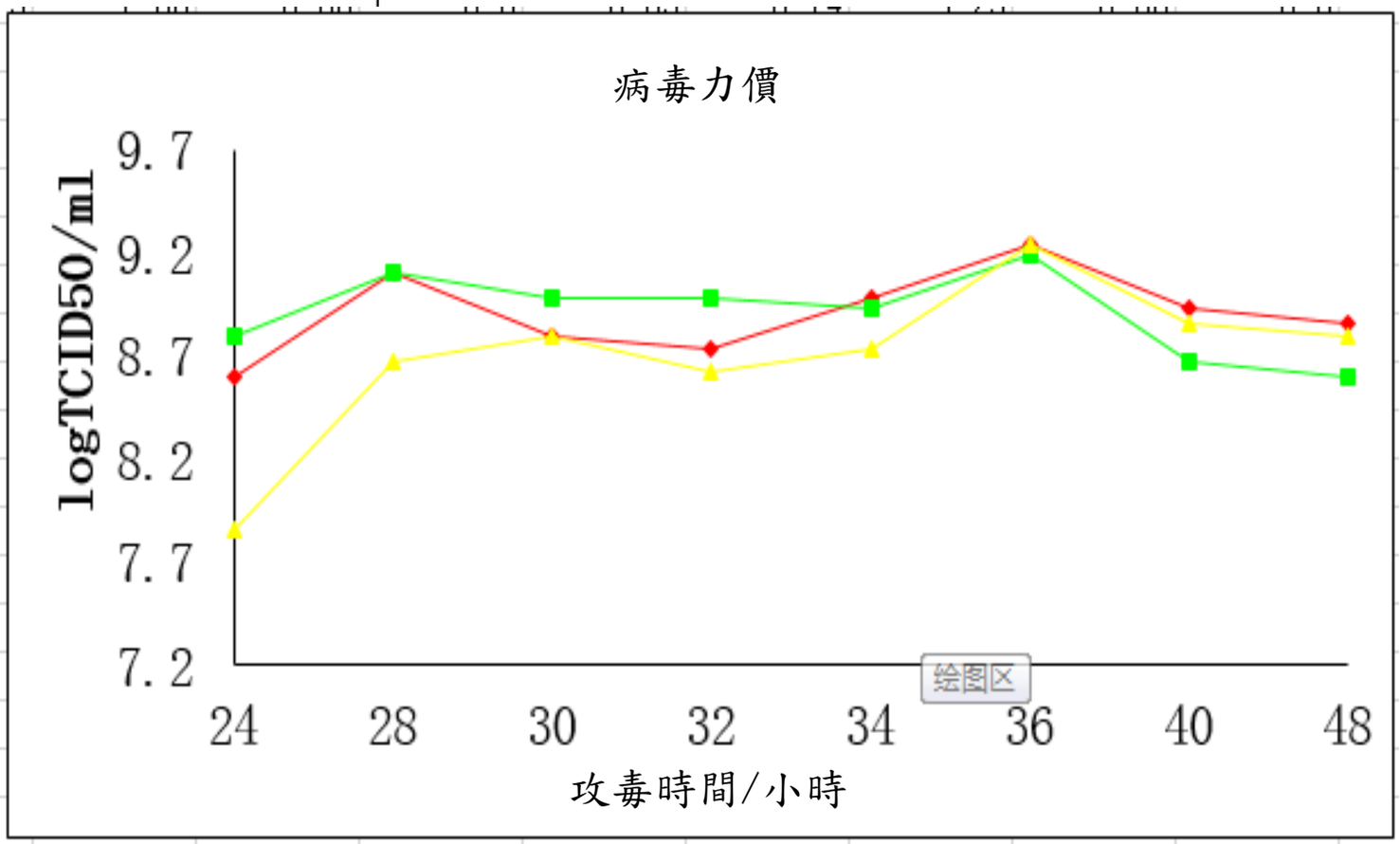




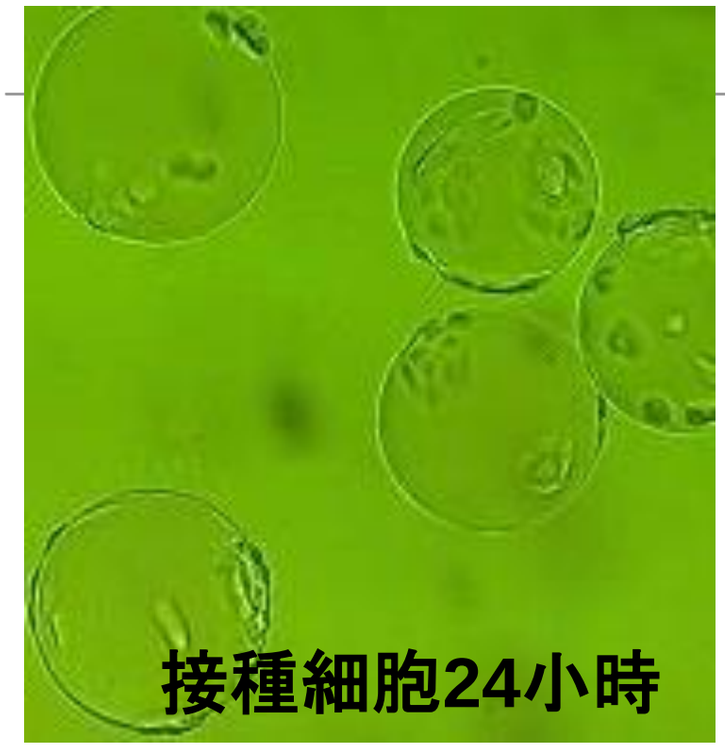


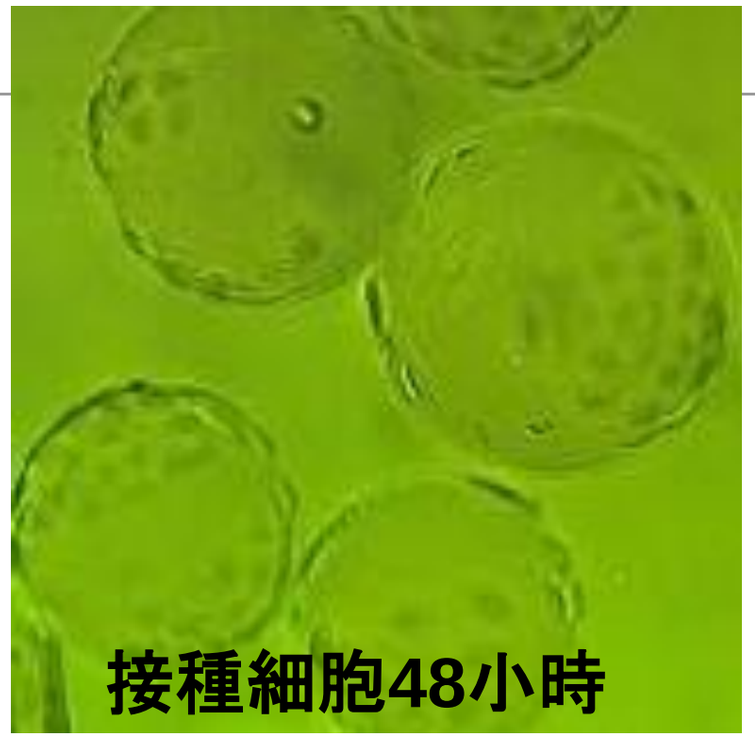

細胞密度1×10^5cells/ml
40
30
20
10
0
3.2
5.6
12.7
26.5
34.8
35.2
34.6
0
24
48
72
96
120
144
小時

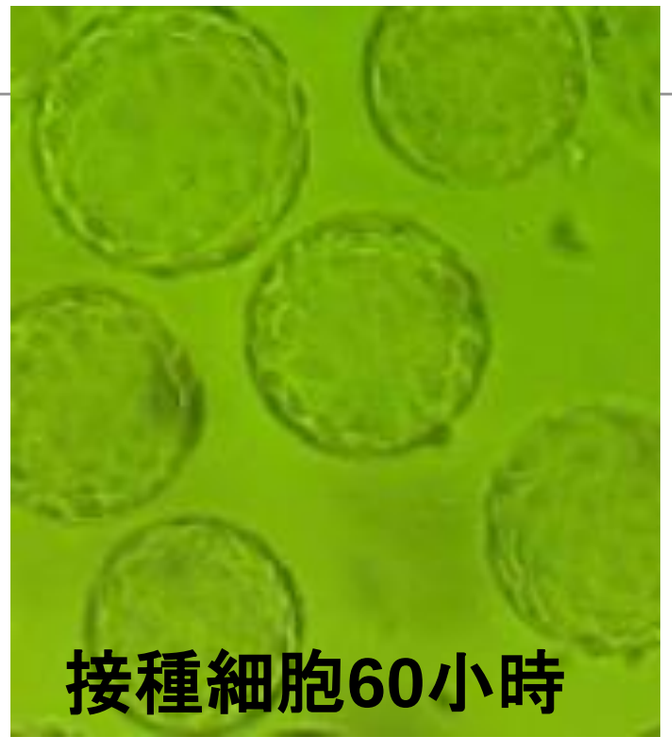
3g/L載體

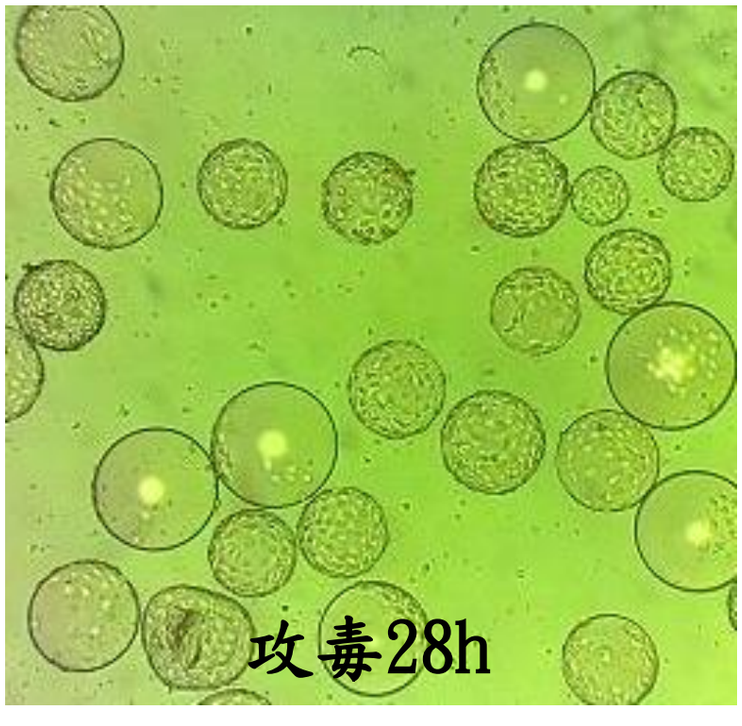

病毒力價
logTCID50/ml
9.7
9.2
8.7
8.2
7.7
7.2
绘图区
24
28
30
32
34
36
40
48
攻毒時間/小時

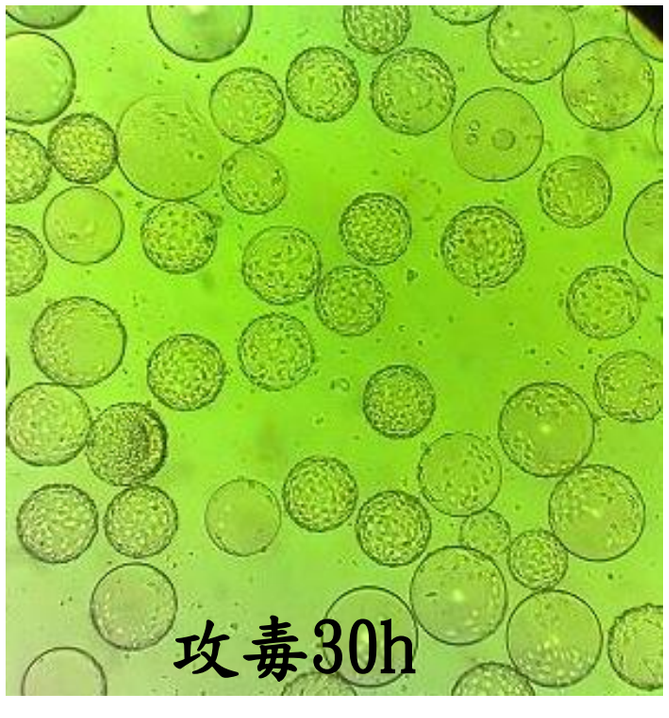
Vero細胞 PEDV

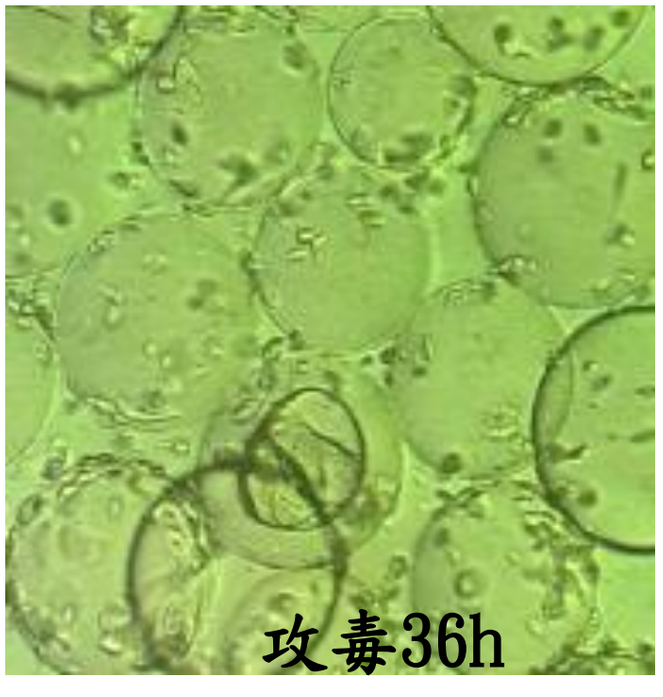




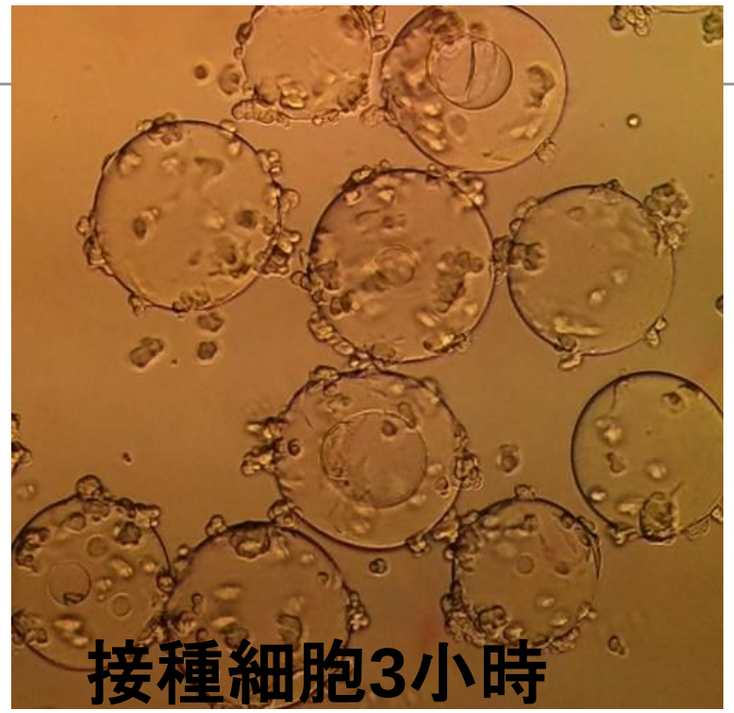


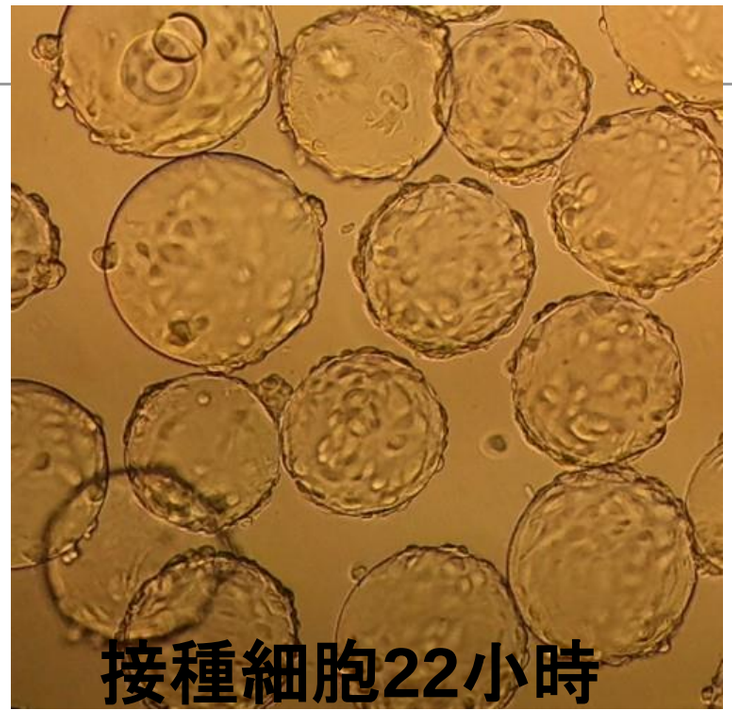


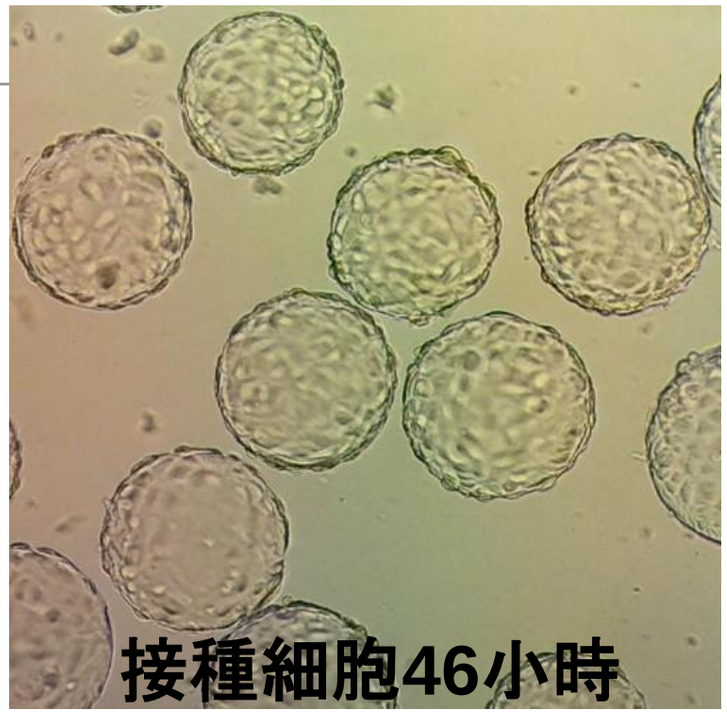


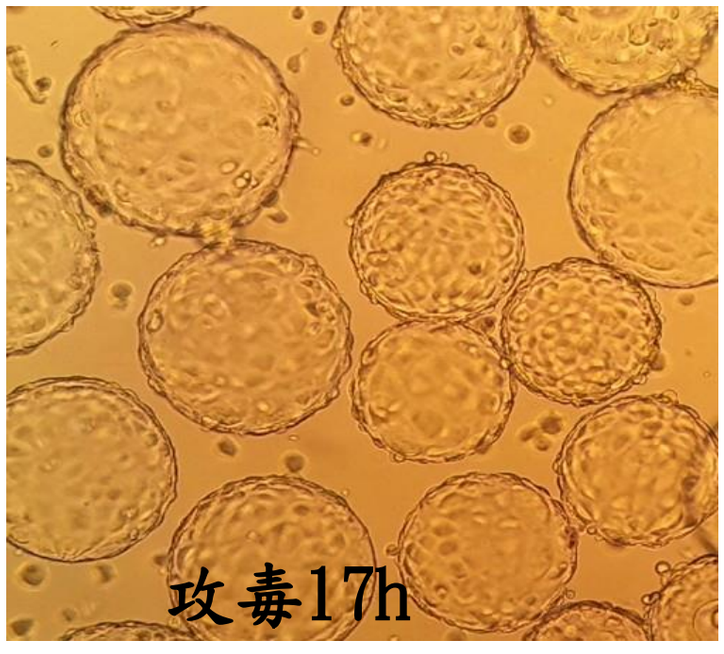


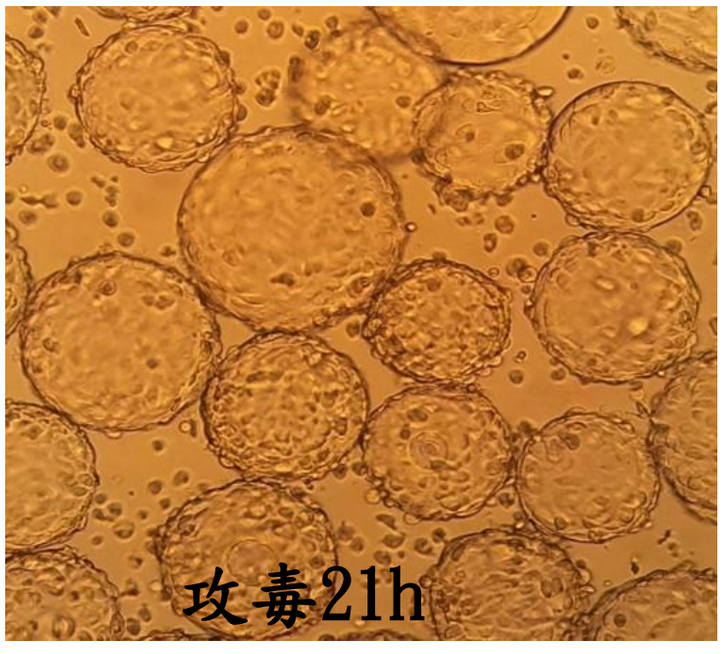
ST細胞/ TGEV

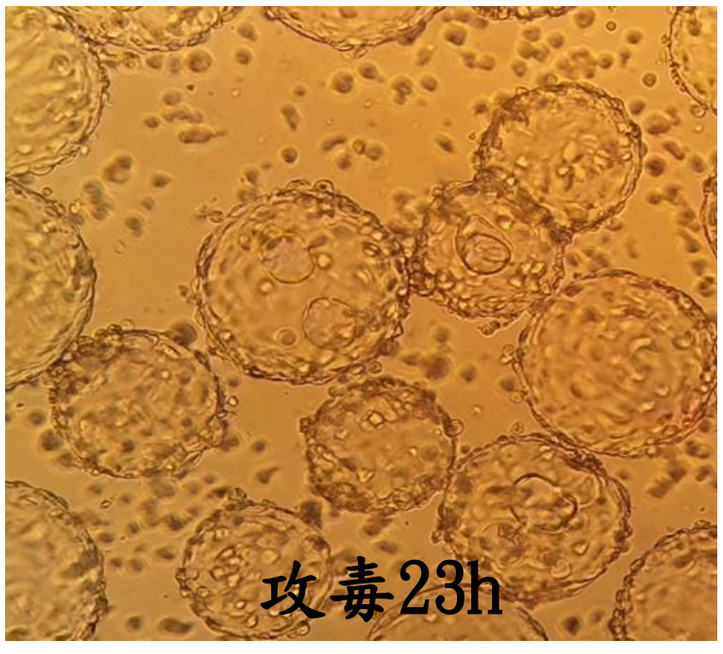












全懸浮式細胞培養

技術優點:

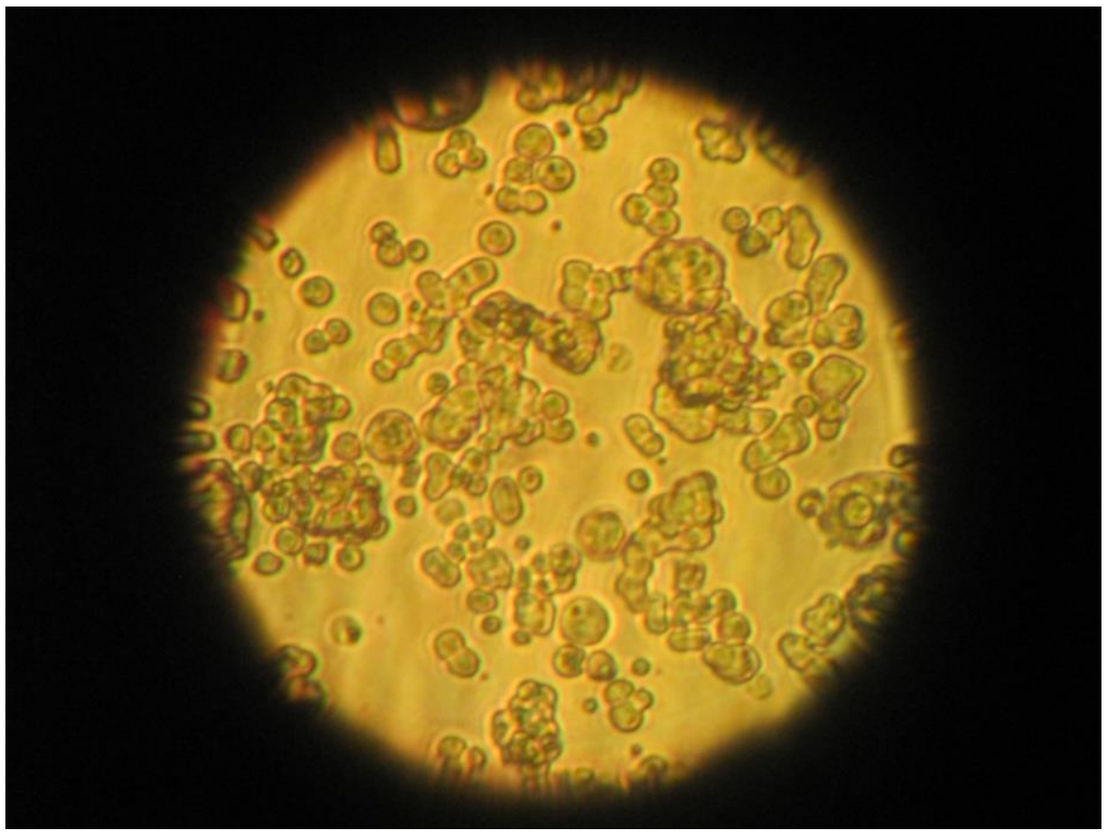
1. 不需使用任何載體或微粒子
2. 以電腦監控製程，可隨時調整培養條件

簡易實驗流程規劃:

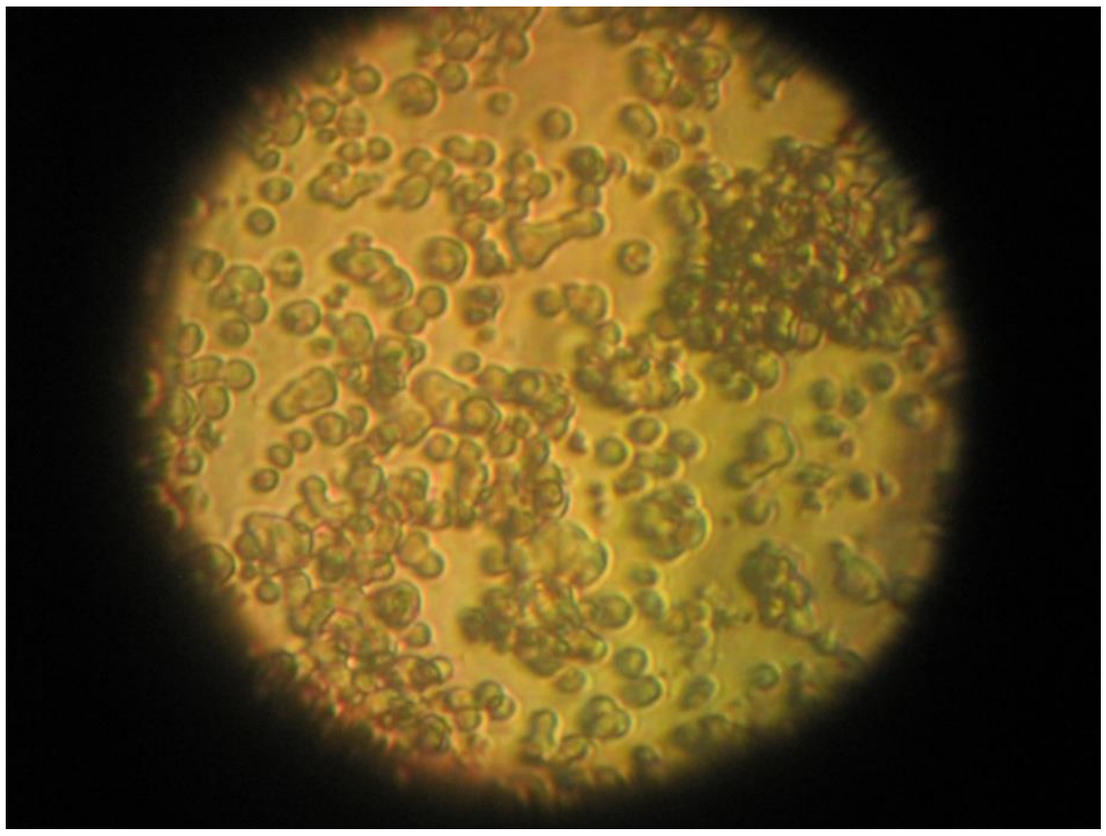
轉瓶培養之BHK21細胞 → 計算細胞數 → 將 1x10^5 cells /mL 放入已加入 5 L MEM 之生物反應器 → 以 37˚C懸浮方式生長 → 96 小時內快速增殖，最高大約可達 6x10^6 cells/ mL

世界全懸浮培養現況

- 可運用細胞株： BHK21、MDCK、VeroE6、ST、Marc145、293T、CHO



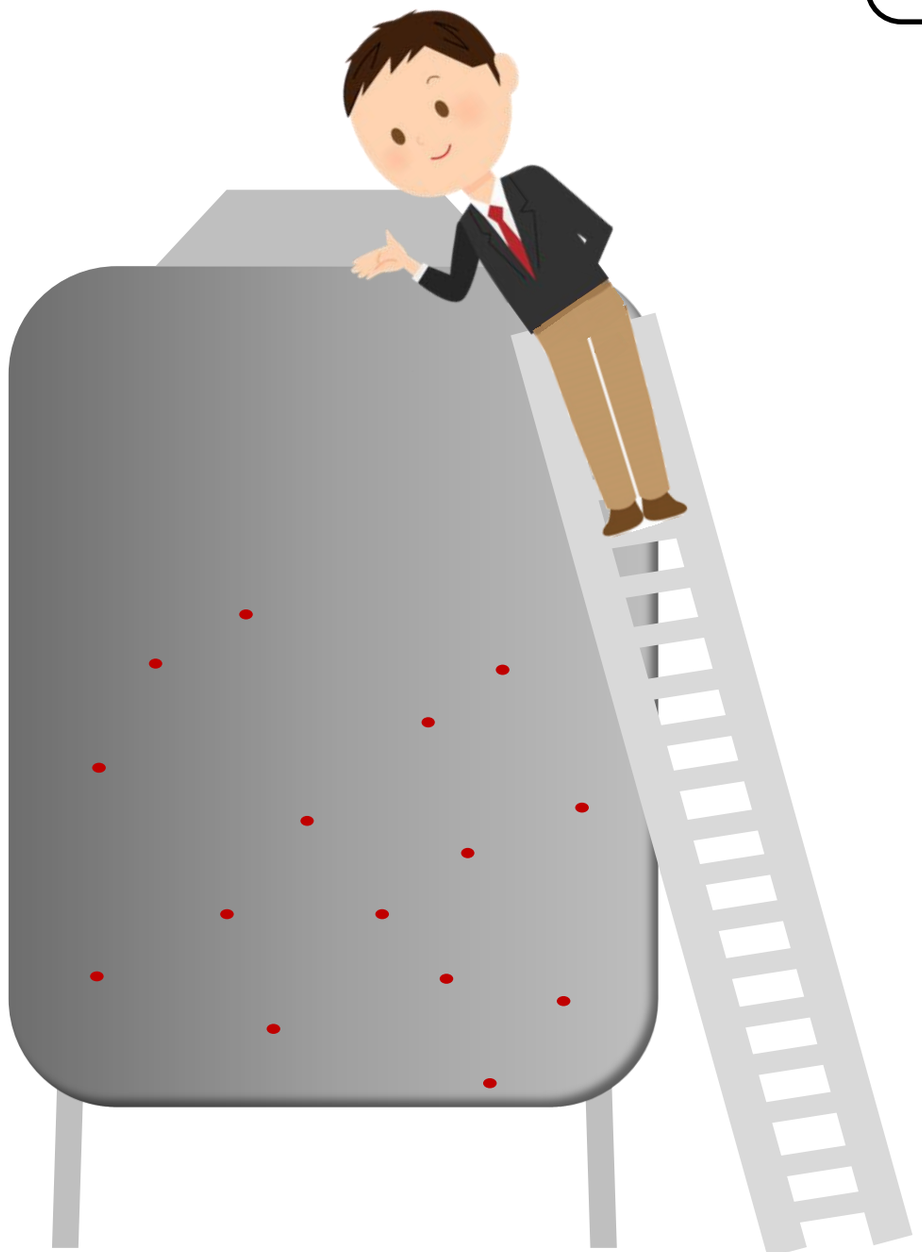
MDCK



Vero-E6

高密度生產

量產



高密度生產

Cell Culture Media

- A. Bulk ions - Na, K, Ca, Mg, Cl, P, Bicarb or CO_2
  B. Trace elements - iron, zinc, selenium
  C. Sugars - glucose is the most common
  D. Amino acids - 13 essential
  E. Vitamins
  F. Choline, inositol (cell structure and membrane integrity)
  G. Serum
  H. Antibiotics - although not required for cell growth,

  antibiotics are often used to control the growth of bacterial

  and fungal contaminants.

無血清培養基開發策略

血清作用	血清組分	替代策略
營養	氨基酸、多肽、維生素、微量元素等	• 優化培養基營養成分
生長調節	生長因數、激素（如胰島素）等	• 添加發酵生產的胰島素、IGF、EGF等 • 馴化細胞至低生長因數、甚至不需生長因數狀 • 優化培養基組分，添加鋅離子等
運輸、調節蛋白	白蛋白、鐵轉運蛋白、粘連蛋白等	• 添加發酵生產的相應蛋白 • 馴化細胞至低蛋白、甚至不需蛋白狀態 • 優化培養基組分，如使用檸檬酸運輸鐵
保護	細胞活性劑、蛋白酶抑制劑、抗氧化劑等	• 優化培養基組分

選擇適合的生長培養基/適應選擇的生長培養基?

分組	A	B	C	D	E	F
成份	MEM	MEM TPCK-TV	MEM TTV	DMEM	DMEM TPCK-TV	DMEM TPCK-TV
Cells	Vero	Vero	Vero	Vero	Vero	Vero
Ct-1	14.18	10.99	11.43	13.03	10.05	10.66
Ct-2	15.12	11.22	11.59	13.98	10.44	11.03
Note					***	

世界疫苗研發重點

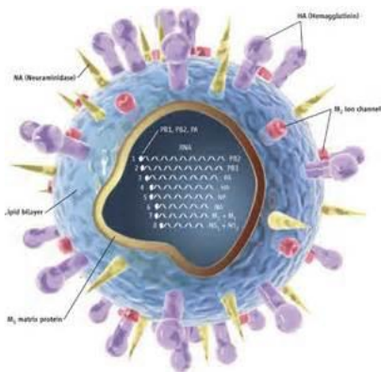
- 開發新疫苗

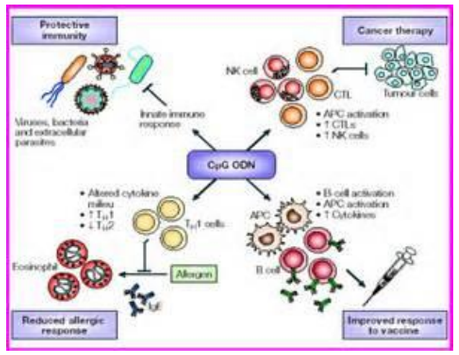
- 改變疫苗生產的方式
- 對抗不斷變異的病原
- 降低生產成本
- 最佳品質管控
- 符合國際趨勢

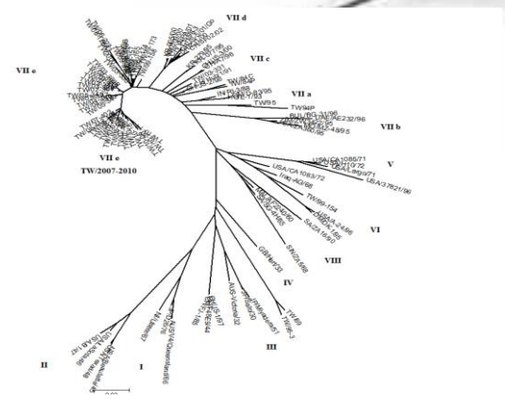
符合國際規範
適用於全世界
具備國際競爭力

1.開發新疫苗……

- 環境因素
- 流行毒株
- 病原特性
- 作用機轉
- 生產方法
- 成本考量
- 免疫方式
- 效果

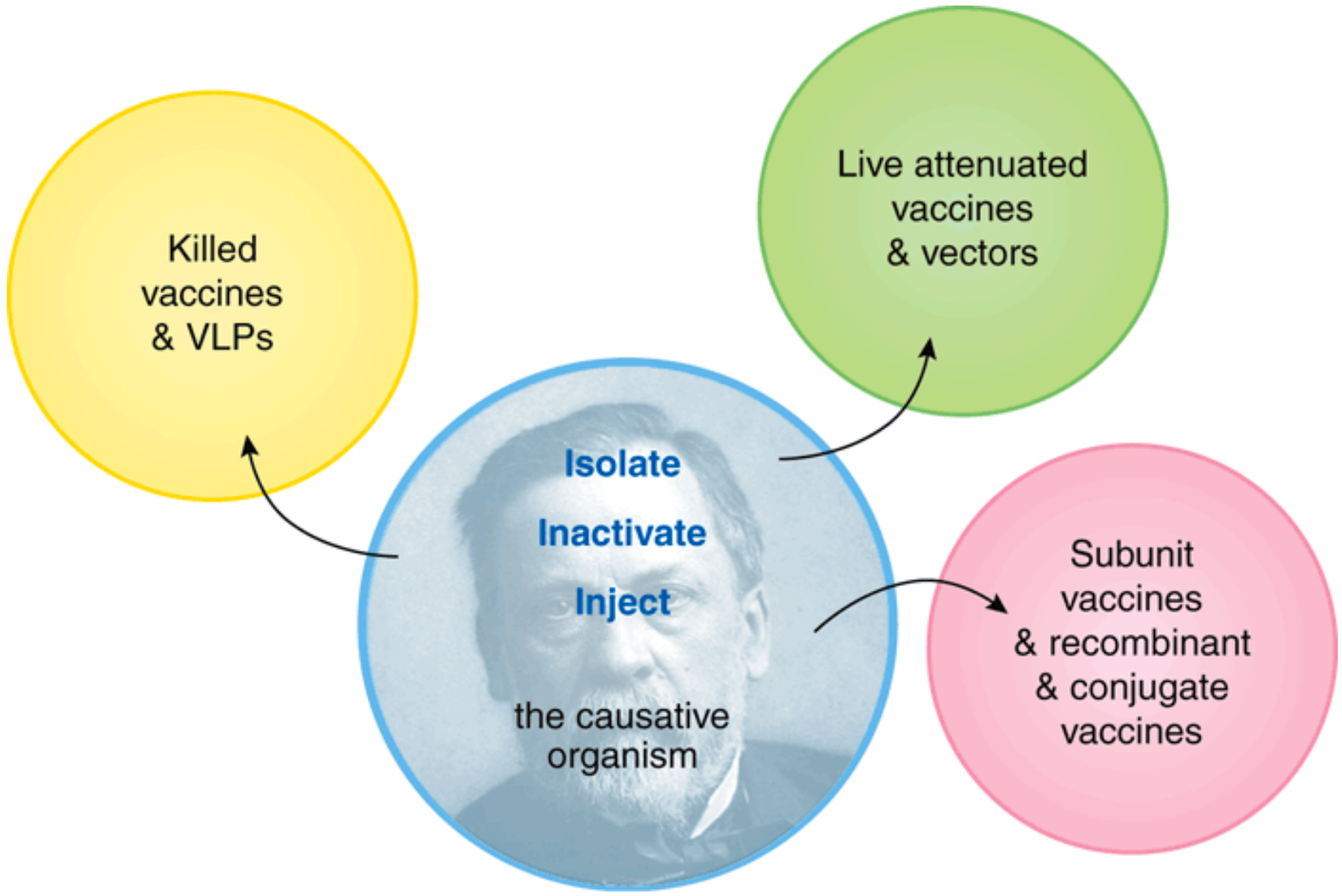






疫苗種類

- 活毒疫苗
- 不活化疫苗
- 次單位疫苗
- DNA疫苗
- 載體疫苗



2.改變疫苗生產的方式……

2L 反應槽

300萬劑/反應槽

=40X

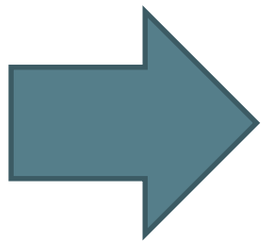
120L Space

7.5萬劑/Bottle

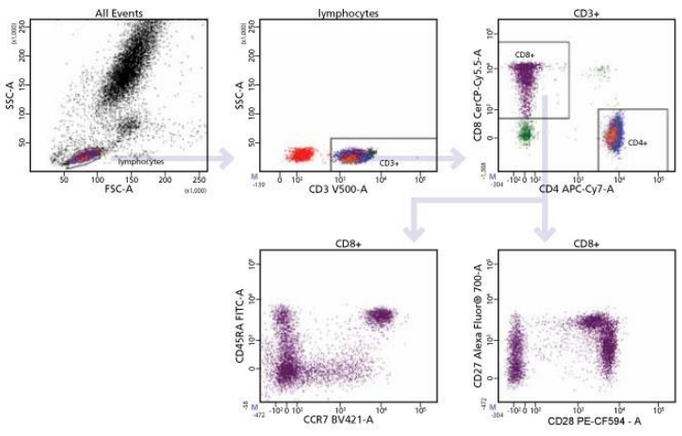
=4000X

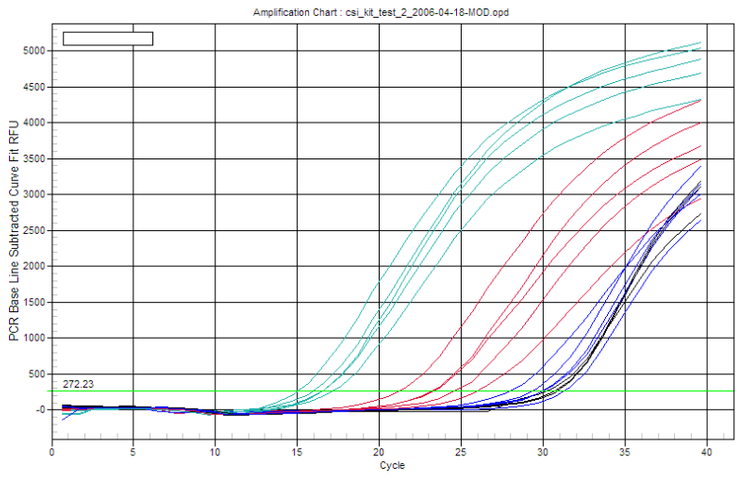
1000劑/兔子

3.最佳品質管控……

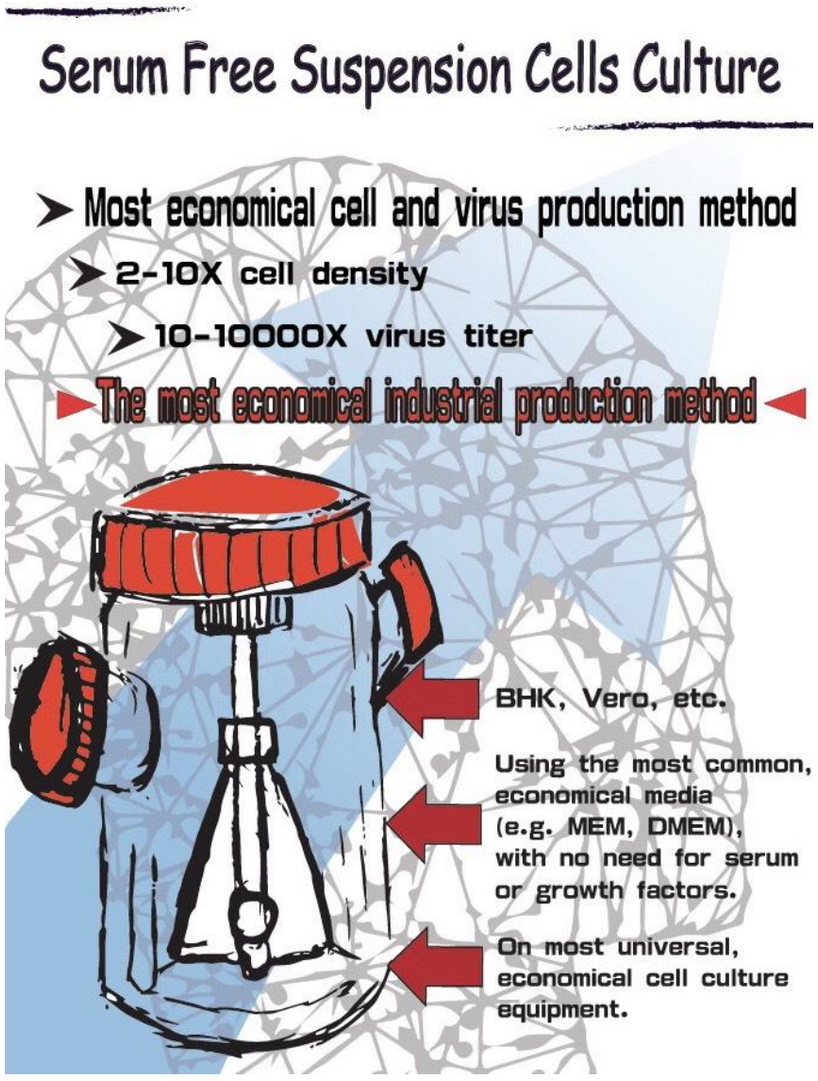


廠房設計
動線改善
法規說明
文件建立
確效作業
種源系統建立



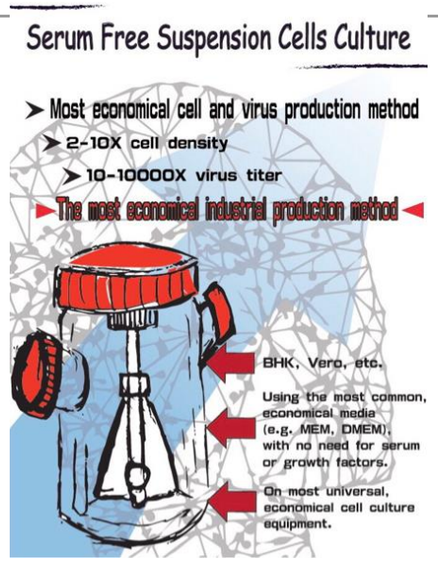


4.降低生產成本……


Serum Free Suspension Cells Culture
➤ Most economical cell and virus production method
➤ 2-10X cell density
➤ 10-10000X virus titer
The most economical industrial production method
BHK, Vero, etc.
Using the most common, economical media (e.g. MEM, DMEM), with no need for serum or growth factors.
On most universal, economical cell culture equipment.


Serum Free Suspension Cells Culture
➤ Most economical cell and virus production method
BHK, Vero, etc.
MICROSTIRRER
Magnetic Stirrer
VELP SCIENTIFICA
Stirrer

5.符合國際趨勢
(動物福祉)



=40X

300萬劑/反應槽(2L)

Roller Bottle R-850

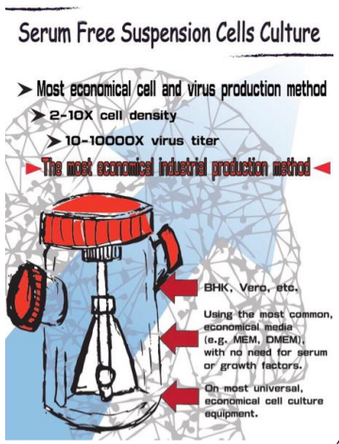
120L Space

7.5萬劑/Bottle

=4000X

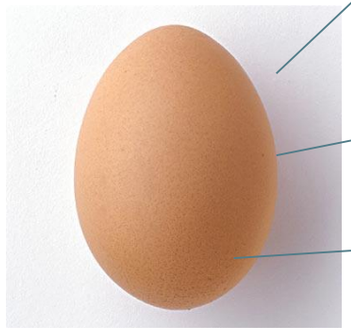
1000劑/兔子

(環境保護減碳)



=285X

反應槽(2L)



總重量約 80 g

尿囊液約 7 g ➡ 疫苗生產

廢棄物約 73 g ➡ 滅菌 ➡ 廢棄物處理

5.符合國際趨勢
(產業升級)

PIC/S GMP
Taiwan FDA

cGMP
CURRENT GOOD MANUFACTURING PRACTICE

WHO-GMP

廠房設計
動線改善
法規說明
文件建立
確效作業
種源系統建立

aaalac
INTERNATIONAL

動物房設計
實驗動物倫理

ISO 17025
ACCREDITED
LABORATORY
QUALITY MANAGEMENT SYSTEM

TAF
財團法人
全國認證基金會

實驗室品質認證

Thanks!