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Rapid Fluorescent Focus Inhibition Test (RFFIT):

*Strains of virus
*Infection of cell culture
*Harvesting & Titration of virus

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OIE Virtual Training Series on Rabies Serology for SAARC Region
11-13 October 2021, 2 PM Japan Time (GMT+9)

Rapid Fluorescent Focus Inhibition Test (RFFIT)

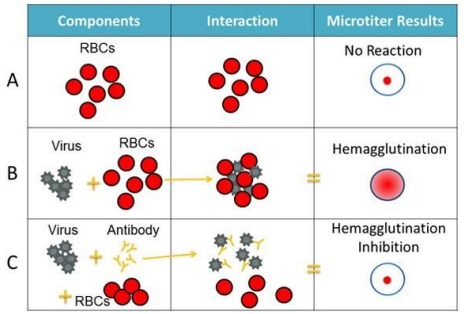
- RFFIT- virus neutralization (VN) test
  - Detecting rabies virus neutralizing antibodies
- Residual virus detected using fluorescence microscope

Application:

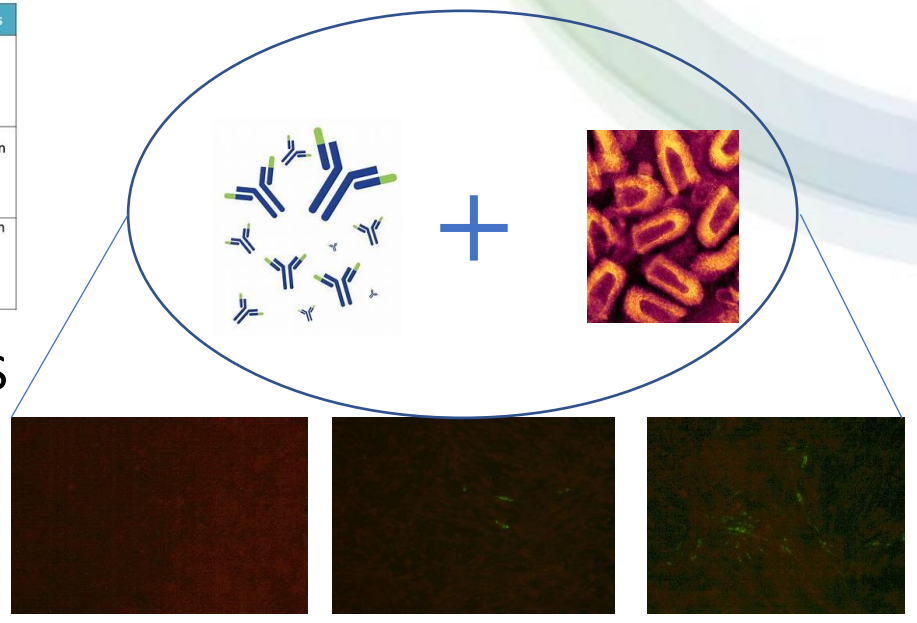
- Determining immune responses to vaccination
- International Pet travel
- Monitoring mass vaccination campaigns in dogs

Principle of RFFIT

HI



RFFIT



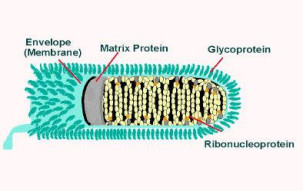
Similar to Haemagglutination inhibition

- Two fold dilutions of **test serum** **+** fixed amount (100 TCID 50)CVS
- Neutralization detected by inoculating cell culture.
- Presence or absence of virus - DFA.

- Recording highest serum dilution at which 100% of the challenge inoculum neutralized – No fluoresence.
- Titre of neutralizing antibody in test serum (in IU/ml) obtained by comparison with the titre of ref. serum.

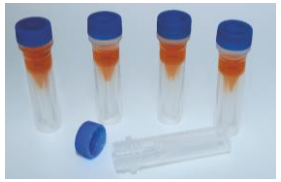
Strains of virus, infection of cell culture, harvesting and titration of virus used for RFFIT

Strains of rabies virus used for RFFIT-

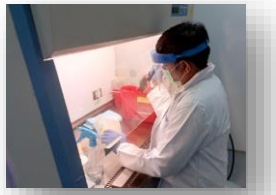


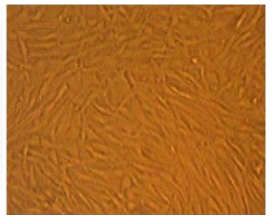
1.PV 3462 (Dr. Larghi's) strain -Pasteur Institute of India, Coonoor

2.CVS-11 strain - NIMHANS, Bengaluru



- Seed virus stored at -80±-2 °C
- Aliquot from seed lot grown, harvested and titrated.
- **Avoid repeat freezing - thawing**





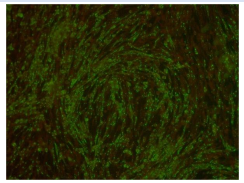
Infection of cell culture

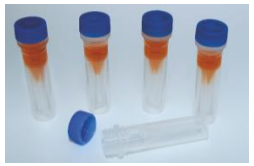
- BHK 21 cells with 80 % monolayer - used for inoculation of seed virus
- Monolayer seeded with Rabies virus - 37 °C in 5 % CO_2 - viral adsorption for 90 mins
- Remove viral inoculum – Add growth medium (GM) incubated at 37 °C in CO_2 for 48 hrs.

Estimation of virus inoculum to be seeded:

$$\text{Volume of virus stock to be added} = \frac{\text{multiplicity of infection (MOI)} \times \text{number of cells}}{\text{Virus titre}}$$

BHK cells infected with rabies virus at a multiplicity of infection (MOI) of 0.1.





Harvesting of virus

Virus harvested in a cryovial, aliquoted and stored in – 80 °C .

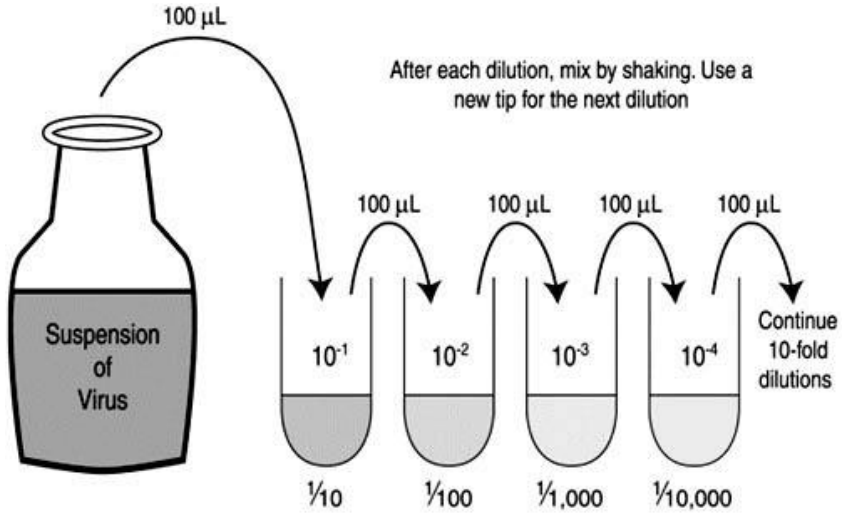
Titration of the harvested virus - To estimate the infectious unit of virus

Procedure:

Harvested virus 10 fold serially diluted GM

(Log dilutions from 10^{-1} to 10^{-6})

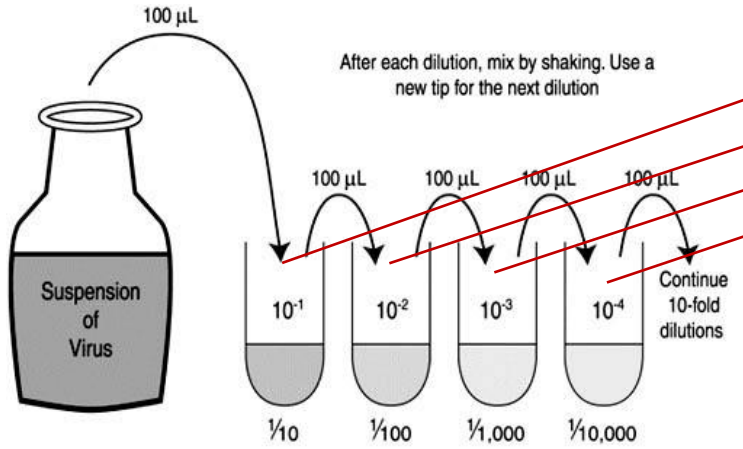
Tube no.	1	2	3	4	5	6
Virus	100µl	10x serial dilution	→			
Diluent	900µl	900µl	900µl	900µl	900µl	900µl
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}



Five wells for each dilution (5 replicates).

Virus dilutions 10^{-1} to 10^{-6} added into columns 1-6 (100 µL)

Table 1: Layout of microtitre plate for virus titration



	1 10^{-1}	2 10^{-2}	3 10^{-3}	4 10^{-4}	5 10^{-5}	6 10^{-6}						
A							CC	VC				
B							CC	VC				
C							CC	VC				
D							CC	VC				
E							CC	VC				

50 µl of BHK- 21

↓

Cell control (CC)- 50 µL BHK 21 with 100 µL of GM

↓

Virus control (VC) - 50 µL of BHK 21 +100 µL of neat virus with GM

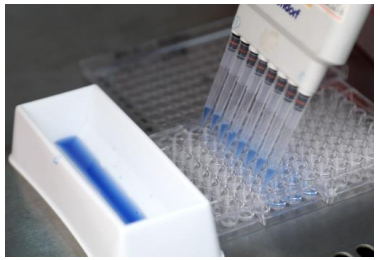
↓

5 % CO_2 at 37 °C ± 1 °C for 48 hrs.

Acetone fixation: - Supernatant removed - 100 µL of 70 % chilled acetone added.

-20 ^{o}C freezer for 60 min.

Addition of FITC conjugate: acetone removed & plate air dried for 5 min.



50 µL of (1:15 ± 1:5) anti-rabies **N-protein Mab FITC conjugate**

37 ^{o}C for 60 ± 5 minutes.

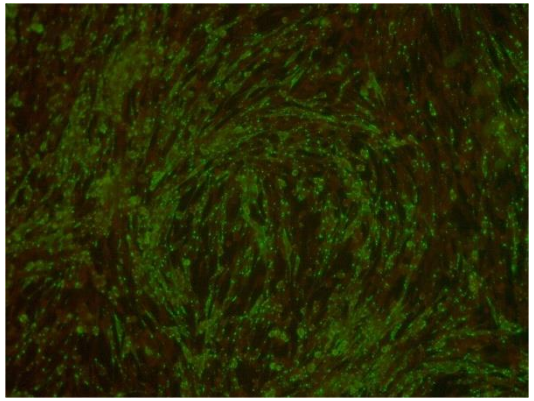
Washing of plates: Contents discarded and washed (1x PBS) twice

Observation (10X and 20X objectives)

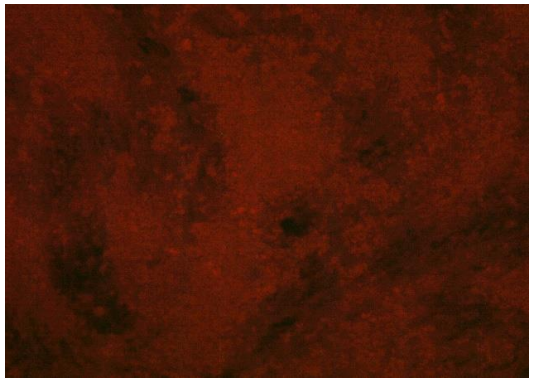
All the fields in each well

Presence or absence of viral inclusions as apple green fluorescent particles.

The observations at various dilutions of virus documented (Table 1): two faculties to approve



Virus control



Cell Control

Interpretation of results:

Reed-Muench method (1938) in terms of $TCID_{50}$.

Virus dilution	Infection ratio	Infected	Uninfected	Accumulated values		Infection ratio (I/I + U)	Percent (I/I+ U)*100
				Infected (I)	Uninfected (U)		
10^{-1}	/5						
10^{-2}	/5						
10^{-3}	/5						
10^{-4}	/5						
10^{-5}	/5						
10^{-6}	/5						

The 50% end point considered for infection. The Proportionate Distance (PD) is calculated as below:

$$\text{Proportionate Distance (PD)} = \frac{\text{Infectivity above 50 \% - 50}}{\text{Infectivity above 50\% - Infectivity below 50 \%}}$$

log dilution above 50 per cent considered. Hence, the 50 per cent end point is calculated in the following way:

(log ID50) = (log dilution above 50 per cent) + (Proportionate Distance × log dilution factor)

$TCID_{50}$ calculated as 10^{-x}/ 0.1 ml

End point dilution : Highest dilution that infects 50 per cent of the test units inoculated - **one $TCID_{50}$.**

100 $TCID_{50}$ = $10^{-(x-2)}$/ 0.1 ml

Antilog (x-2) is the dilution factor.

Original virus stock is diluted by {antilog (x-2)} times to get 100 $TCID_{50}$ virus.

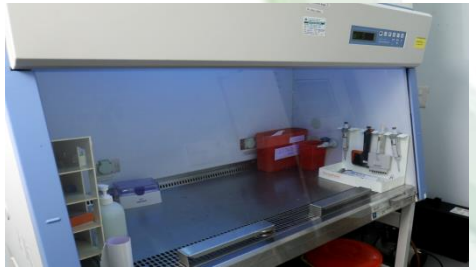
100 $TCID_{50}$ virus used in RFFIT

Rapid Fluorescent Focus Inhibition Test (RFFIT)- Methodology

Materials and equipment

- Test serum samples
- BHK-21 cell lines
- Titrated virus lots of PV 3462 (Dr. Larghi's) strain or CVS-11 of rabies virus
- 96-well tissue culture plate
- GM
- PBS pH 7.2±0.2
- Variable channel Micro-pipettes (1-10 µl, 20-100 µl and 20-1000 µl)
- Sterile disposable microtips (1-10µl, 20-200µl and 1000µl)
- Sterile Petri plates
- BSC
- Reference anti-rabies serum: ERIG
- CO_2 incubator
- Inverted Microscope with fluorescence attachment
- Anti-rabies nucleoprotein *Ig*G FITC conjugate
- 70 % Chilled acetone with double distilled water

RFFIT - Procedure



Preparation of BSC

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Heat inactivation of serum samples at 56±2 °C for 30-32 min.

↓

RFFIT microtiter plate layout

Sample No	1	2	3	4	5	6	7	8	9	10	Ref.S	Controls
A	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2 Ref.S	VC
B	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4 Ref.S	VC
C	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8 Ref.S	VC
D	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16 Ref.S	VC
E	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32 Ref.S	CC
F	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64 Ref.S	CC
G	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128 Ref.S	CC
H	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256 Ref.S	CC

100 µL of GM to sample, Ref. and VC wells

200 µL of GM added to CC wells .

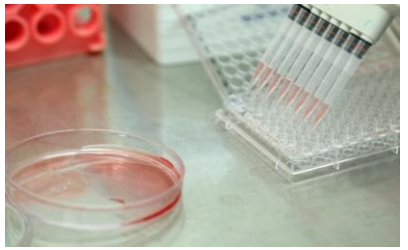
100 µL of test serum added - to the first well (1:2) and mixed.

Mixed, 100 µL from first well transferred to second well (1:4) and similar serial dilutions upto 8th well (1:256)

100 µL discarded from the eighth well.

Similarly , 2 fold dilutions of reference serum (eRIG) made.

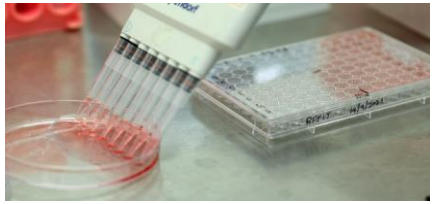
Sample No	1	2	3	4	5	6	7	8	9	10	Ref.S	Controls
A	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2 Ref.S	VC
B	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4 Ref.S	VC
C	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8 Ref.S	VC
D	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16 Ref.S	VC
E	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32 Ref.S	CC
F	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64 Ref.S	CC
G	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128 Ref.S	CC
H	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256 Ref.S	CC



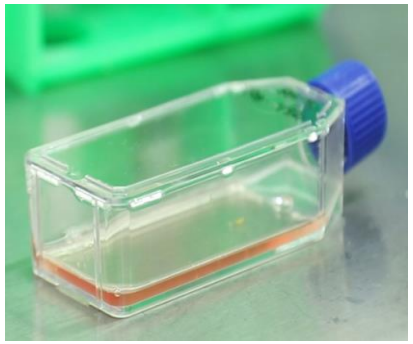
100µL of 100 $TCID_{50}$ of virus added to all wells – Except CC wells

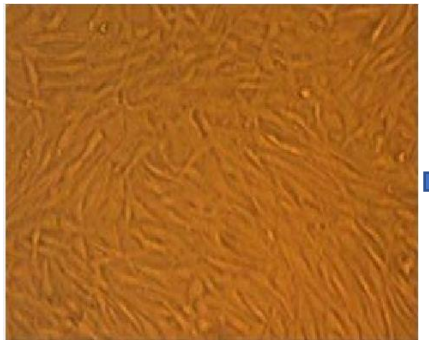
5% CO_2 for 60-65 min at 37±1 ^oC

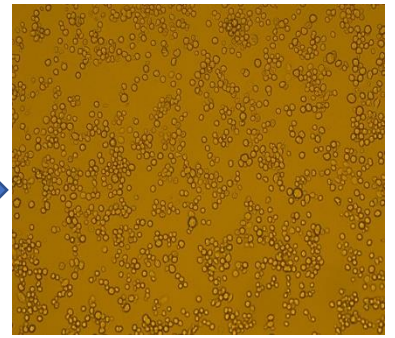
BHK-21 (80-90%) in T-25 flask trypsinized by using trypsin-EDTA in GM.

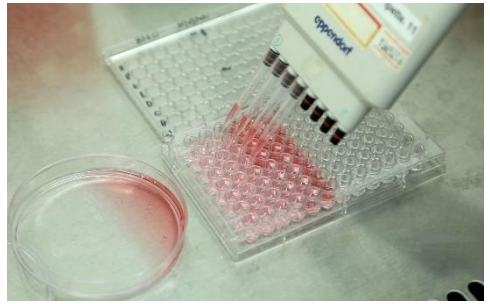


50 µL of BHK 21 added to each well









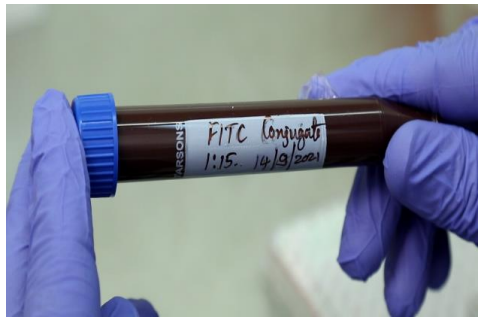
Incubated at 5% CO_2 for 48 hours at 37±1 ^oC

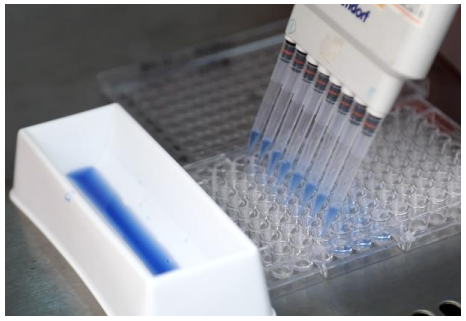
Acetone fixation: supernatant removed - 100 µL of 70 % chilled acetone added.

-20 ^{o}C freezer for 60 min.

Addition of FITC conjugate: acetone removed & plate air dried for 2-5 minutes.

50 µL (1:15 ± 1:5) anti-rabies **N-protein Mab FITC conjugate** added.

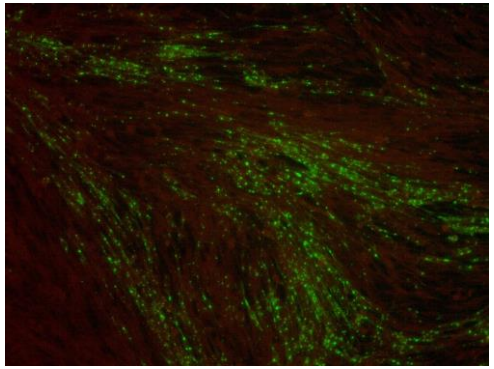




37 ^{o}C for 60 ± 5 min.

Washing : Contents discarded and washed (1x PBS) 3x

Observed under 10X and 20X objectives of flu.



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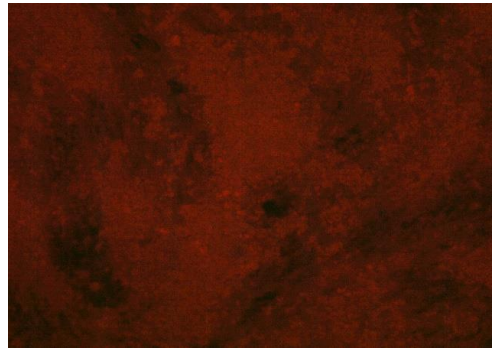
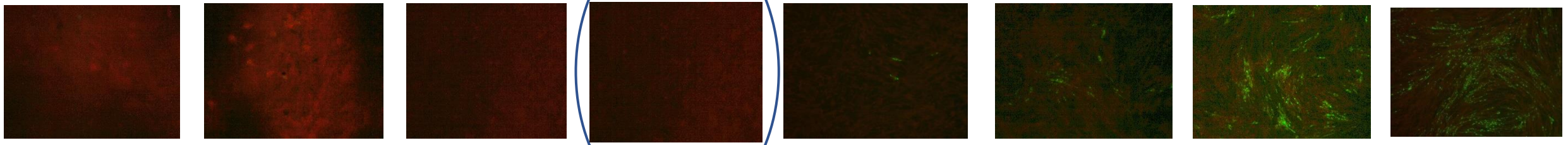


VC

CC

Sample reading



1:2 serum dilution

1:4 serum dilution

1:8 serum dilution

1:16 serum dilution

1:32 serum dilution

1:64 serum dilution

1:128 serum dilution

1:256 serum dilution

Result: 4.0 IU/ml