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Version 1

RSV Expansion and Titration V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

Protocol to expand and titrate RSV

Materials

Trypsin-EDTA, 0.05% Thermo Fisher Scientific Cat #25300054

PBS Gibco Cat #20012027

TISSUE CULTURE MEDIUM (TCM): Filter through 0.2 µm filter

	Component	Amount	Conc. Supp.	Product information
	DMEM	500 mL		Gibco Cat. # 11965092 (#11965118-cs)
	Gentamicin	500 µL	50µg/mL	Gibco Cat #15750060 (#15750078-pk) – 50 mg/mL
	Sodium Pyruvate	5.0 mL	1mM	Corning Cat #25-000-CI – 100mM
	L-Glutamine	5.5 mL	2 mM	Sigma Aldrich Cat #G7513 – 200mM
	FBS	50 mL	10%	

INFECTION MEDIUM – RSV: Filter through 0.2 µm filter

	Component	Amount	Conc. Supp.	Product information
	DMEM	500 mL		Gibco Cat. # 11965092 (#11965118-cs)
	Gentamicin	500 µL	50µg/mL	Gibco Cat #15750060 (#15750078-pk) – 50 mg/mL
	Sodium Pyruvate	5.0 mL	1mM	Corning Cat #25-000-CI – 100mM
	L-Glutamine	5.5 mL	2 mM	Sigma Aldrich Cat #G7513 – 200mM
	FBS	10 mL	2%	

Disposable cell scraper Fisher Scientific Cat #08-100-241

Ultra-Clear™ centrifuge tubes, 25 × 89 mm Beckman Coulter Cat #344058

Hanks' balanced salt solution (HBSS) Gibco Cat #14-025-092

Crystal violet Sigma Cat #C0775

CRYSTAL VIOLET SOLUTION

- **STOCK:** 1% Crystal Violet powder in 20% C₂H₅OH solution



To make 100mL STOCK:

1g Crystal Violet

20mL absolute $\text{C}_2\text{H}_5\text{OH}$ (ethanol)

80mL water

■ **Working Dilution:**

40mL STOCK

80mL CH_3OH (methanol)

180mL dd H_2O



Cell Preparation

- 1 Seed 1×10^7 HEp2 cells per T225 flask the night before
Typically, seed 1-2 flasks for a seed stock and 4-8 flasks for a working stock

RSV Infection

- 2 Wash cells once with PBS
- 3 For LD stock: Infect HEp2 cells with RSV-LD at an MOI of 0.01
For HD stock: Infect HEp2 cells with RSV-LD at an MOI of 4

Note

LD infection: stock containing low levels of non-standard viral genomes
HD infection: stock containing high levels of non-standard viral genomes

Dilute virus in RSV infection media

Add  4-5 mL of diluted virus to each T225 flask

- 4 Incubate at  37 °C for  01:00:00 rocking flasks every  00:15:00 to maintain an even virus distribution and avoid the cells drying out

1h

- 5 After  01:00:00 incubation, add  25 mL RSV infection media to each flask

- 6 Incubate at  37 °C

Harvest

- 7 Check the progress of infection
LD - harvest at 4-5dpi, may or may not see cell death
HD - harvest at 2dpi, or until half the cells are dead
If the virus has fluorescence, use that to ensure productive infection is visible before harvesting
- 8 Scrape the cells and collect them together with the culture supernatant



9 Centrifuge at 1180 rpm for  00:05:00 at  4 °C and collect the supernatant into fresh tubes and place on ice

5m

10 Add  2-3 mL of supernatant back to the cell debris. Keep the remaining supernatant on ice

11 Freeze (in a mixture of dry ice+ethanol) / thaw (in  37 °C water bath) the cell debris 3 times, vortex in between each thaw and freeze

Note

This step is to help aid release the virions from the cell surface and help increase the titer

12 Centrifuge cell debris at 1180 rpm for  00:05:00 and only take the supernatants and combine with the supernatant on ice. Discard the debris.

5m

13 For seed stocks (P1), you can stop here and aliquot the virus supernatant and titer. Once the virus is aliquoted, snap freeze in dry ice + ethanol before storing in  -80 °C
For working stock (P2), it's recommended to follow steps 14-17 to achieve a higher titer stock

14 Ultra-centrifuge the supernatants  59000 x g, 4°C, 02:30:00 using a SW 32Ti Rotor and Ultra-Clear™ 25×89 mm centrifuge tubes to concentrate the working virus stock

2h 30m

15 Discard the supernatant and lightly wash the pellet with  1 mL HBSS twice without disturbing the pellet

16 Resuspend pellet in RSV infection media

 200-800 µL per T225 flask

*Resuspension volume can be adjusted based on desired titer and may depend on the strain of RSV

17 Aliquot (100 µL/vial) and snap freeze in dry ice + ethanol before storing in  -80 °C

TCID50 - Titration of RSV

18 Prepare 96-well plates with HEp2 cells (2×10^6 cells/10mL of TCM). Seed  100 µL per well the night before. Cells should be 80-90% confluent at the time of infection



- 19 Prepare a dilution plate with serial 1/10 dilutions of the virus in triplicate. Remember to include a positive control and a negative (media alone). Change tips between each dilution
- 20 Remove media from cells and wash once with PBS
- 21 Transfer  25 μL of virus dilution/well. Start with the lowest dilution - changing tips is not necessary
- 22 Incubate at  37 °C for  01:00:00 rocking flasks every  00:15:00 to maintain an even virus distribution and avoid the cells drying out
- 23 Add  175 μL of RSV infection media to each well
- 24 Incubate for 4-5 days and look at CPE or perform the following crystal violet staining
- 25 Discard media into a container containing bleach
- 26 Add  50 μL of crystal violet working solution to each well. Be careful not to touch the bottom of the wells
- 27 Incubate at  Room temperature for  00:20:00 -  00:30:00
- 28 Discard crystal violet and wash the plate by submerging it upside down in water several times to eliminate the excess crystal violet
- 29 Let plate air dry
- 30 Determine the endpoint dilution by reading the CPE in the monolayer
- 31 Score the titer by determining the last dilution with positive CPE. Score the number of positive wells for that last dilution (number of positive out of three). From this score,

1h

30m

calculate the TCID₅₀/25µL using the following formula, where "x" corresponds to the last dilution with positive CPE:

$$+++ \quad 10^x \text{ TCID}_{50} = 10^{x+0.7}/25 \mu\text{L}$$

$$++- \quad 10^x \text{ TCID}_{50} = 10^{x+0.4}/25 \mu\text{L}$$

$$+-- \quad 10^x \text{ TCID}_{50} = 10^{x-0.1}/25 \mu\text{L}$$

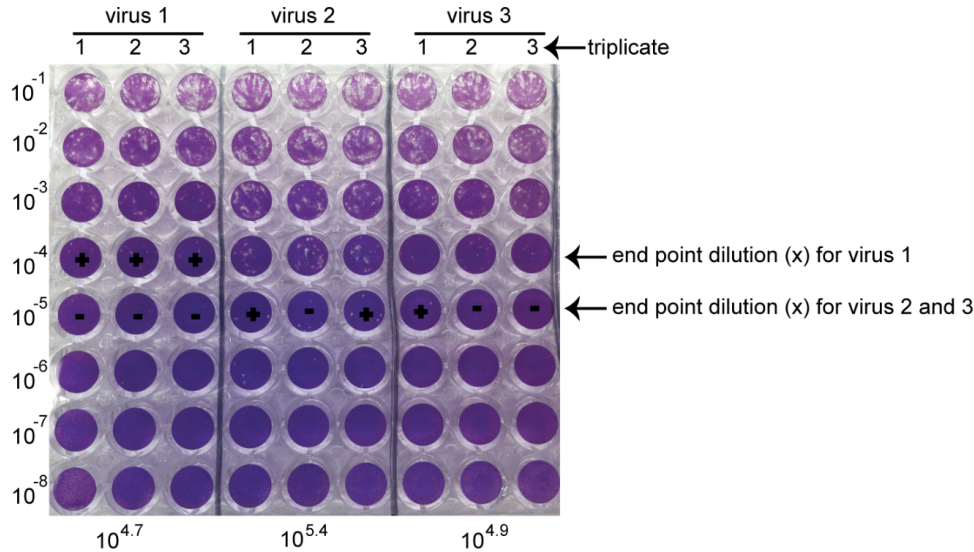


Figure 3 from Preparation of respiratory syncytial virus stocks and purification of defective viral particles. Bio-protocol 6(10): e1820. <http://www.bio-protocol.org/e1820>.

Crystal violet staining of TCID₅₀. The titers from three different viruses were determined by TCID₅₀ as illustrated above. 10⁻⁴ and 10⁻⁵ were the last dilutions with positive CPE for designated virus samples, which were the "x". "+" stands for the positive CPE observed. "-" indicates no CPE observed. Based on the equation above, calculate the final titer of each virus as shown at the bottom of this figure.

Note

For working virus stocks, we typically do 3 independent repeats of the TCID₅₀, then calculate the average for the working virus stock titer that will be used in future experiments



Protocol references

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For details about this protocol, please refer to Preparation of respiratory syncytial virus stocks and purification of defective viral particles. Bio-protocol 6(10): e1820. <http://www.bio-protocol.org/e1820>.