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RSV Expansion, Purification, and Titration

Forked from RSV Expansion and Titration

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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

Ethylenediaminetetraacetic acid disodium salt solution (EDTA) pH 8.0, 0.5M Sigma Aldrich Cat #03690

D-Sucrose (Molecular Biology) Fisher Scientific Cat #BP220-1

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% C_2H_5OH solution

  To make 100mL STOCK:
  1g Crystal Violet
  20mL absolute C_2H_5OH (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 , 5% CO_2 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 , 5% CO_2

Harvest + Purification 2h 10m

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 Prepare PNE buffer:
50 mL PBS supplemented with 200 µL 0.5M EDTA

(adjust volumes depending on how many flasks are being harvested)

9 Prepare 33% sucrose:
16.5 g sucrose dissolved in 50 mL PNE buffer
(adjust volumes depending on how many flasks are being harvested)

10 Scrape the cells and collect them together with the culture supernatant

11 Centrifuge at 1180 rpm for 00:05:00 at 4 °C and collect the supernatant into fresh tubes and place on ice 5m

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

13 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

14 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

15 Dilute the virus mixture by adding additional RSV infection media - up to 1:1

Note

A 1:1 mix is a suggestion, but not required. The more dilute the stock is, the better it will be able to separate through the sucrose cushion. However, dilution volume can be determined by calculating the total volume from all flasks and how many tubes/rounds of ultra-centrifugation will be done. For example, one round of ultra-centrifugation using all 6 tube holders will allow for a maximum of 180 mL virus mixture. Therefore, multiple rounds of ultra-centrifugation will be required if harvesting 8 flasks.

16 Add 8 mL of the 33% sucrose solution to each Ultra-Clear™ 25×89 mm centrifuge tube to prepare the sucrose cushion

17 Add 30 mL of virus mixture to each tube. Slowly add by pipetting on the side of the tube to try to avoid any mixing with the sucrose layer. Place any remaining virus mixture on ice.

18 Ultra-centrifuge the supernatants in the Ultra-Clear™ 25×89 mm centrifuge tubes at 28000 rpm, 4°C, 02:00:00 using a SW 32Ti Rotor and Optima XPN-100 Ultracentrifuge to concentrate the working virus stock 2h

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

Note

If the pellet is very small or loose, the washing can be omitted to avoid losing particles. However, attempt to remove all liquid as best as possible before resuspending the pellet.

20 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
*Can resuspend in PBS instead of infection media if needed

21 Repeat steps 16-20 if multiple rounds of ultracentrifugation are required.

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

TCID50 - Titration of RSV

23 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

24 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

25 Remove media from cells and wash once with PBS

26 Transfer 25 µL of virus dilution/well. Start with the lowest dilution - changing tips is not necessary

27 Incubate at 37 °C, 5% CO_2 for 01:00:00 rocking flasks every 00:15:00 to maintain an even virus distribution and avoid the cells drying out

1h

28 Add 175 µL of RSV infection media to each well

29 Incubate for 4-5 days and look at CPE or perform the following crystal violet staining

30 Discard media into a container containing bleach

31 Add 50 µL of crystal violet working solution to each well. Be careful not to touch the bottom of the wells

32 Incubate at Room temperature for 00:20:00 - 00:30:00

30m

33 Discard crystal violet and wash the plate by submerging it upside down in water several times to eliminate the excess crystal violet

34 Let plate air dry

35 Determine the endpoint dilution by reading the CPE in the monolayer

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

"+++" 10^{X} $TCID_{50}$ = $10^{X+0.7}$/25 µL

"++-" 10^{X} $TCID_{50}$ = $10^{X+0.4}$/25 µL

"+--" 10^{X} $TCID_{50}$ = $10^{X-0.1}$/25 µL

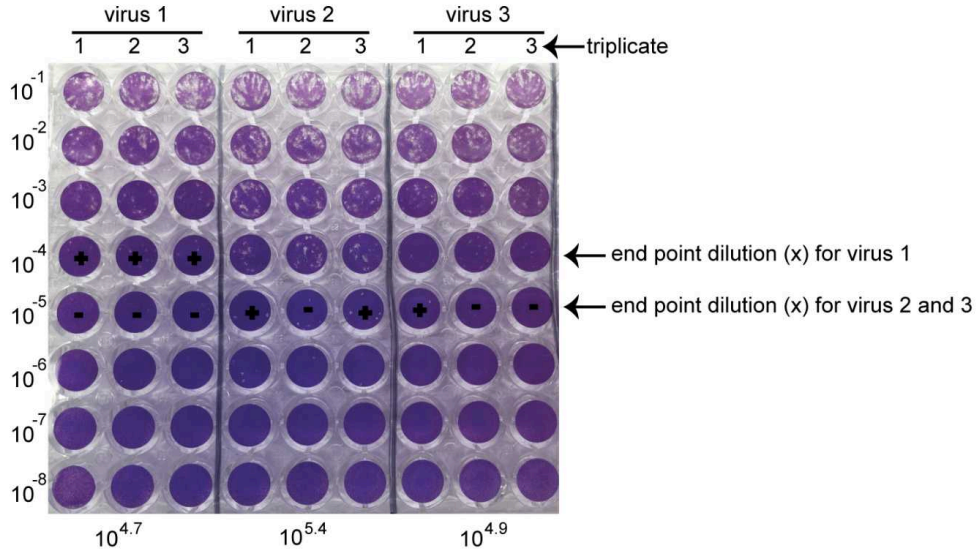

Figure 3 from Preparation of respiratory syncytial virus stocks and purification of defective viral particles. Bio-protocol 6(10): e1820. http://www.bio-protocol.org/e1820.
Crystal violet staining of $TCID_{50}$. The titers from three different viruses were determined by $TCID_{50}$ as illustrated above. 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.