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Title: Targeting Cas9 to Perform Rescue Experiments in Stably Transduced Knockout HEK293 Cells

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No.**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

Current Protocol Length

Number of Steps: 23

Number of Shots: 54

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

Authors: The answers are slightly edited in accordance with the journal guidelines.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **John Sharifi:** We study virus-host dynamics, using CRISPR-Cas9 gene knockouts to understand how cellular proteins regulate viral replication.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **John Sharifi:** This protocol applies to stable CRISPR-Cas9-expressing knockout cell lines, including adherent, suspension, and transfection-refractory systems requiring transgene-driven rescue analyses.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **John Sharifi:** This protocol enables rescue assays where the transgene restoration reverses the knockout phenotype, confirming the protein's cellular function.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **John Sharifi:** The main challenge is optimizing the multiplicity of infection to balance delivery efficiency against insertional mutagenesis and homeostatic compensation.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **John Sharifi:** This framework also enables phenotypic exploration of protein variants without confounding background signal from the native wild-type protein.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.

Provide the **full name and position** of the author delivering the testimonial.

Please answer the testimonial question live during the shoot, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your JoVE article address?

- 1.6. **John Sharifi, Principal Investigator, Le Moyne College:** (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

What is the key takeaway you hope viewers remember after watching your video article?

- 1.7. **John Sharifi, Principal Investigator, Le Moyne College:** (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer's NOTE: For the interview section we did not film any testimonial statements

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Production and Harvest of Cas9-Specific gRNA Lentivirus

Demonstrator: Lauren Postell

2.1. To begin, seed 2.5×10^6 HEK293 (Heck-293)-FT (F-T) cells in a 10-centimeter tissue culture-treated dish containing complete DMEM [1]. Incubate at 37 degrees Celsius in a humidified incubator containing 5% carbon dioxide for 12 hours [2-TXT].

2.1.1. WIDE: Talent adding the HEK293FT cells into a 10 centimeter tissue culture-treated dish.

2.1.2. WIDE: Talent placing the tissue culture-treated dish into the incubator. **TXT: See text protocol for media and reagent compositions**

2.2. Dilute 20 micrograms of the total plasmid DNA in the sterile molecular biology-grade water to a final volume of 450 microliters in a 1.5 milliliter microcentrifuge tube [1-TXT]. Then, add 50 microliters of 2.5 molar calcium chloride [2], followed by 500 microliters of 2× HEPES-buffered saline at pH 7.05 to obtain a final volume of 1 milliliter [3]. Mix the calcium phosphate transfection solution thoroughly by gentle pipetting [4].

2.2.1. Talent adding the plasmid DNA to a 1.5 milliliter microcentrifuge tube.

TXT: Cas9 gRNA1_pLentiGuide-Hygro: 5 µg

pLP1: 5.6 µg

pLP2: 3.8 µg

pLP/VSVG: 5.6 µg

2.2.2. Talent adding 50 microliters of 2.5 molar calcium chloride to the microcentrifuge tube.

2.2.3. Talent adding 500 microliters of 2× HEPES-buffered saline to the microcentrifuge tube.

2.2.4. Talent gently pipetting the calcium phosphate transfection solution to mix thoroughly.

2.3. Once the HEK293FT cells reach at least 60% confluence, add 1 milliliter of the calcium phosphate transfection mixture dropwise [1]. Gently rock the culture dish to distribute the transfection complexes evenly [2]. Incubate the cells at 37 degrees Celsius for 12

hours [3].

2.3.1. Talent adding the calcium phosphate transfection mixture dropwise to the HEK293FT cell culture.

2.3.2. Talent gently rocking the culture dish.

2.3.3. WIDE: Talent placing the dish in the incubator.

2.4. After incubation, aspirate and discard the culture medium [1]. Wash the cells once with 10 milliliters of sterile PBS [2].

2.4.1. Talent aspirating and discarding the culture medium.

2.4.2. Talent adding the cells with 10 milliliters of room-temperature sterile 1× PBS.

2.5. Discard the PBS and add 10 milliliters of fresh complete DMEM [1]. Then, return the cells to the incubator and allow the lentiviral particles to accumulate in the culture medium for 36 hours [2].

2.5.1. Talent discarding the PBS and adding the fresh room-temperature complete DMEM to the culture dish.

2.5.2. WIDE: Talent placing the dish in incubator. **Videographer's NOTE: 2.5.2 has a tail slate at the end of the shot**

2.6. Carefully transfer the lentivirus-containing supernatant to a sterile 15-milliliter conical tube without disturbing the HEK293FT cell monolayer [1]. Centrifuge the supernatant at 1,000 *g* for 10 minutes at room temperature to pellet residual cells [2].

2.6.1. Talent carefully transferring the lentivirus-containing supernatant from the HEK293FT producer cells into a sterile 15 milliliter conical tube.

2.6.2. WIDE: Talent placing the 15 milliliter conical tube into the centrifuge.

Authors: This step is moved here for better flow

3. Transduction of HEK293ΔCUL4B Cells with Cas9-Specific gRNA Lentivirus

Demonstrators: John Sharifi

3.1. Seed 1.0×10^6 of HEK293ΔCUL4B (*HEK-293-Delta-Cul-4B*) adherent target cells in a 10-centimeter tissue culture-treated dish [1-TXT] and incubate for 12 hours before

lentiviral transduction [2]. After discarding the media, transfer 7 milliliters of the clarified lentiviral supernatant to the dish [3].

3.1.1. Talent adding the cells to the dish. **TXT: CUL4B: Cullin-4B**

3.1.2. Talent placing the plate in the incubator.

3.1.3. Talent transferring 7 milliliters of the clarified lentiviral supernatant to the 10 centimeter dish containing the HEK293ΔCUL4B cells.

3.2. Incubate the transduced cells for 24 hours to permit the complete lentiviral transduction and integration [1]. After discarding the viral supernatant, wash the cells once with 10 milliliters of room-temperature sterile 1× PBS [2]. Aspirate and discard the PBS [3].

3.2.1. Talent placing the cells in the incubator.

3.2.2. Talent washing the cells with 10 milliliters of room-temperature sterile 1× PBS. **Videographer's NOTE: 3.2.2 and 3.2.3 were merged into one continuous shot**

3.2.3. Talent aspirating and discarding the PBS.

3.3. Add 10 milliliters of fresh room-temperature complete DMEM [1]. Then return the cells to the incubator and culture them for 4 days to allow transgene expression and Cas9-mediated disruption [2].

3.3.1. Talent adding 10 milliliters of fresh room-temperature complete DMEM to the culture dish.

3.3.2. Talent placing the cells in the incubator. **Videographer's NOTE: 3.3.2 was not shot but previous incubator shots was filmed several takes, so use it here**

4. Hygromycin Selection of Cas9-Specific gRNA Transductants

Demonstrator: Lauren Postell

4.1. Aspirate and discard the culture medium from the target cells [1]. Wash the cells once with 10 milliliters of room-temperature sterile 1× PBS [2]. After discarding PBS, add 2 milliliters of a sterile room-temperature trypsin-EDTA solution to the culture dish and incubate the cells for 5 minutes to promote detachment [3].

4.1.1. WIDE: Talent aspirating and discarding the culture medium from the HEK293ΔCUL4B cells.

4.1.2. Talent washing the cells by adding 10 milliliters of room-temperature sterile 1× PBS.

- 4.1.3. Talent adding a sterile room-temperature trypsin-EDTA solution to the culture dish and placing the dish aside.
 - 4.2. Then, add 8 milliliters of complete DMEM to neutralize the trypsin [1].
 - 4.2.1. Talent adding room-temperature complete DMEM to the culture dish.
 - 4.3. Transfer the cell suspension to a sterile 15-milliliter conical tube [1]. Triturate the cell suspension 15 to 20 times to thoroughly dissociate the cellular aggregates [2].
 - 4.3.1. Talent transferring the cell suspension to a sterile 15 milliliter conical tube.
 - 4.3.2. Talent triturating the cell suspension repeatedly.
 - 4.4. Next, centrifuge the cells at 200 *g* for 10 minutes at room temperature [1]. After centrifugation, aspirate and discard the supernatant without disturbing the cell pellet [1]. Resuspend the cell pellet in 10 milliliters of complete DMEM supplemented with 250 micrograms per milliliter of hygromycin B [2].
 - 4.4.1. WIDE: Talent placing the 15 milliliter conical tube into the centrifuge.
 - 4.4.2. Talent aspirating and discarding the supernatant while leaving the cell pellet undisturbed.
 - 4.4.3. Talent resuspending the cell pellet in complete DMEM supplemented with 250 micrograms per milliliter of hygromycin B.
- Videographer's NOTE:** 4.4.1 and 4.4.2 were merged into one continuous shot
- 4.5. Prepare a T150 tissue culture-treated flask containing 20 milliliters of complete DMEM supplemented with 250 micrograms per milliliter of hygromycin B [1]. Transfer the 10 milliliter cell suspension into the flask and gently rock the flask to distribute the cells evenly [2]. Then, incubate the cells for 3 days [3].
 - 4.5.1. Talent adding culture medium to T150 flask under a hood.
 - 4.5.2. Talent transferring the cell suspension into the prepared T150 tissue culture-treated flask and gently rocking the flask.
 - 4.5.3. Talent placing the flask in the incubator.
 - 4.6. On day 3 of the selection, replace the selection medium with 30 milliliters of fresh complete DMEM supplemented with 250 micrograms per milliliter of hygromycin B [1]. Continue incubating the cells until the surviving cell monolayer reaches approximately

90 percent confluence, typically after an additional 4 days [2].

4.6.1. Talent replacing the selection medium in the T150 tissue culture-treated flask with fresh medium containing hygromycin B.

4.6.2. Talent placing the flask in the incubator.

5. Preparing Calcium Phosphate Transfection Mixtures

5.1. Prepare the calcium phosphate transfection mixtures containing 0, 0.5, 1, or 2 micrograms of CUL4B (*Cull-4-B*) plasmid DNA [1-TXT]. Add the empty pCMV vector to bring each mixture to a total of 10 micrograms of plasmid DNA [2].

5.1.1. Talent adding plasmid to a 1.5 ml tube. **TXT: Plasmid: pCMV-FLAG-CUL4B**

5.1.2. Talent adding the empty pCMV vector to adjust each mixture to a total of 10 micrograms of plasmid DNA.

Videographer's NOTE: 5.1.1 and 5.1.2 were merged into one continuous shot

5.2. Dilute each DNA mixture in sterile molecular biology-grade water to a final volume of 180 microliters [1]. Then, add 20 microliters of 2.5 molar calcium chloride, followed by 200 microliters of 2× HEPES-buffered saline at pH 7.05 [3]. Mix each calcium phosphate transfection solution thoroughly by gentle pipetting [4].

5.2.1. Talent adding the DNA mixture and the sterile molecular biology-grade water to a 1.5 milliliter microcentrifuge tube. **Videographer's NOTE: 5.2.1 and 5.2.2 were merged into one continuous shot**

5.2.2. Talent adding 20 microliters of 2.5 molar calcium chloride to the microcentrifuge tube.

5.2.3. Talent adding 200 microliters of 2× HEPES-buffered saline to the microcentrifuge tube.

5.2.4. Talent gently pipetting each calcium phosphate transfection solution to mix thoroughly.

6. Validation of Cas9 Clearance and In Trans CUL4B Rescue

Demonstrators: John Sharifi and Nader Elturk

- 6.1. Seed the early-passage target cells stably expressing a Cas9-targeting guide RNA into 4 wells of a 6-well tissue culture-treated plate at a density of 2.5×10^5 cells per well [1].
 - 6.1.1. Talent seeding the HEK293ΔCUL4BΔcas9 cells into 4 wells of a 6-well tissue culture-treated plate. **TXT: Target cells: HEK293^{ΔCUL4BΔcas9}**
- 6.2. Then, seed the HEK293ΔCUL4B (*Heck-293-delta-Cull-4-B*) cells into 4 wells of a separate 6-well tissue culture-treated plate [1]. Seed the parental HEK293 cells into at least 1 additional well to serve as a control [2]. Incubate all cultures for 12 hours to allow cell attachment and proliferation [3].
 - 6.2.1. Talent seeding the HEK293ΔCUL4B cells into a separate 6-well tissue culture-treated plate.
 - 6.2.2. Talent seeding the parental HEK293 cells into a control wells.
 - 6.2.3. WIDE: Talent placing the cells in the incubator.
- 6.3. Next, add 200 microliters of each transfection mixture dropwise to the designated well containing the delta-Cas9 target cells [1-TXT]. Change the pipette tips between the samples to prevent cross-contamination [2].
 - 6.3.1. Talent adding 200 microliters of each transfection mixture dropwise to the designated wells containing the HEK293ΔCUL4BΔcas9 cells.
 - 6.3.2. Talent discarding the used pipette tip and attaching a new one.
- 6.4. Add the remaining 200 microliters of each transfection mixture dropwise to the corresponding control delta-Cul4B cells [1]. Incubate the transfected cultures for 12 hours [2]. After incubation, aspirate the culture medium from each well [3].
 - 6.4.1. Talent adding the remaining 200 microliters of each corresponding transfection mixture dropwise to the designated control wells.
 - 6.4.2. Talent placing the plate in the incubator.
 - 6.4.3. Talent aspirating the culture medium from each well.
- 6.5. Wash the cells once with 1 milliliter of sterile 1× PBS [1]. After discarding the PBS, add 2 milliliters of fresh complete DMEM to each well [2].
 - 6.5.1. Talent washing the cells by adding 1 milliliter of room-temperature sterile 1× PBS.

6.5.2. Talent adding fresh room-temperature complete DMEM to each well.

6.6. Incubate the cultures for another 24 hours to allow CUL4B expression and downstream responses [1]. Finally, harvest and lyse the cells, then perform Western blotting to assess the expression of CUL4B, Cas9, WDR5, CDC6, and alpha-tubulin [2].

6.6.1. Talent placing the plate in the incubator.

6.6.2. Talent mixing cell pellet with Laemmli sample buffer and then loading a SDS-PAGE gel. **Author's NOTE:** We also added a shot that breaks up: "6.6.2. Talent mixing cell pellet with Laemmli sample buffer and then loading a SDS-PAGE gel.", where we put the samples on a heating block after loading the Laemmli buffer but before loading the SDS-PAGE gel. Show mixing cells with blue buffer, placing the cells on heat block, then loading the SDS PAGE

Results

7. Results

7.1. Survival during the hygromycin B selection confirmed successful lentiviral transduction [1], and the subsequent western blot analysis verified Cas9 ablation [2].

7.1.1. LAB MEDIA: Figure 1.

7.2. CUL4B protein was readily detected in the parental HEK293 cells [1] but was absent from the empty vector-transfected delta-CUL4B and delta-Cas9 cells [2]. Consistent with the loss of CUL4B, WDR5 levels increased [3]. CDC6 levels decreased, confirming the disruption of CUL4B function [4].

7.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the CUL4B band in the parental lane.*

7.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the CUL4B row in the 0 microgram lanes.*

7.2.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the WDR5 row in the parental and 0 microgram lanes.*

7.2.4. LAB MEDIA: Figure 2B. *Video editor: Highlight the CDC6 row in the parental and 0 microgram lanes.*

7.3. Cas9 protein was detected in the delta-CUL4B cells [1] but was undetectable in the parental HEK293 and delta-Cas9 cells, confirming successful Cas9 ablation [2].

7.3.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the Cas9 bands in the Δ CUL4B lanes.*

7.3.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the empty Cas9 row in the parental and Δ CUL4B Δ cas9 lanes.*

7.4. As expected, transfection of the CUL4B expression plasmid into the delta-CUL4B cells resulted in only minimal restoration of CUL4B protein expression [1]. In contrast, the delta-Cas9 cells showed robust CUL4B expression [2], accompanied by reduced WDR5 [3] and increased CDC6 levels [4].

7.4.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the CUL4B band in the rightmost, highest-dose lane of the Δ CUL4B group.*

7.4.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the CUL4B band in the rightmost, highest-dose lane of the Δ CUL4B Δ cas9 group.*

- 7.4.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the WDR5 row in the rightmost, highest-dose lane of the Δ CUL4B Δ cas9 group.*
- 7.4.4. LAB MEDIA: Figure 2B. *Video editor: Highlight the CDC6 row in the rightmost, highest-dose lane of the Δ CUL4B Δ cas9 group.*
- 7.5. Together, these findings demonstrated that Cas9 ablation permitted efficient restoration of CUL4B expression and function [1] by preventing Cas9-mediated cleavage of the rescue plasmid [2].
- 7.5.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the rightmost, highest-dose lane of the Δ CUL4B Δ cas9 group.*

1. Euthanized
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanized>
IPA: /'ju:θəˌnaɪzd/
Phonetic Spelling: YOO·thuh·nyzd
2. Laminar
Pronunciation link: <https://www.merriam-webster.com/dictionary/laminar>
IPA: /'læmənər/
Phonetic Spelling: LAM·uh·ner
3. Blunt dissection
Pronunciation link: No confirmed link found
IPA: /blʌnt dɪ'sekʃən/
Phonetic Spelling: blunt dih·SEK·shuhn
4. Quadriceps
Pronunciation link: <https://www.merriam-webster.com/dictionary/quadriceps>
IPA: /'kwɑːdrəˌseps/
Phonetic Spelling: KWAH·druh·seps
5. Femoris
Pronunciation link: No confirmed link found
IPA: /fi'mɔːrɪs/
Phonetic Spelling: fih·MOR·iss
6. Patellar
Pronunciation link: <https://www.merriam-webster.com/dictionary/patellar>
IPA: /pə'telər/
Phonetic Spelling: puh·TEL·er
7. Microcentrifuge
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊ'sentrɪˌfjuːdʒ/
Phonetic Spelling: my·kroh·SEN·trih·fyooj

8. Mitochondria
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondria>
IPA: /ˌmaɪtəˈkɑːndriə/
Phonetic Spelling: my·tuh·KAHN·dree·uh
9. Mitochondrial
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondrial>
IPA: /ˌmaɪtəˈkɑːndriəl/
Phonetic Spelling: my·tuh·KAHN·dree·uhl
10. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: TRIP·sin
11. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·NAY·tuht
12. Homogenizer
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenizer>
IPA: /həˈmɑːdʒəˌnaɪzər/
Phonetic Spelling: huh·MAH·juh·ny·zer
13. Homogenate
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenate>
IPA: /həˈmɑːdʒəˌneɪt/
Phonetic Spelling: huh·MAH·juh·nayt
14. Respirometry
Pronunciation link: No confirmed link found
IPA: /ˌrɛspəˈrɑːmətri/
Phonetic Spelling: res·puh·RAH·muh·tree
15. Equilibrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibrate>
IPA: /ɪˈkwɪləˌbreɪt/
Phonetic Spelling: ih·KWIL·uh·brayt
16. Pyruvate
Pronunciation link: <https://www.merriam-webster.com/dictionary/pyruvate>
IPA: /paɪˈruːˌveɪt/
Phonetic Spelling: py·ROO·vayt
17. Glutamate
Pronunciation link: <https://www.merriam-webster.com/dictionary/glutamate>
IPA: /ˈgluːtəˌmeɪt/
Phonetic Spelling: GLOO·tuh·mayt
18. Malate
Pronunciation link: <https://www.merriam-webster.com/dictionary/malate>
IPA: /ˈmæləˌteɪt/, /ˈmeɪləˌteɪt/
Phonetic Spelling: MAL·uh·tayt / MAY·luh·tayt

19. Adenosine
 Pronunciation link: <https://www.merriam-webster.com/dictionary/adenosine>
 IPA: /əˈdenəˌsiːn/, /əˈdenəsən/
 Phonetic Spelling: uh·DEN·uh·seen / uh·DEN·uh·suhn
20. Adenosine diphosphate
 Pronunciation link: <https://www.merriam-webster.com/dictionary/adenosine%20diphosphate>
 IPA: /əˈdenəˌsiːn daɪˈfəːsˌfeɪt/
 Phonetic Spelling: uh·DEN·uh·seen dy·FOSS·fayt
21. Oxidative phosphorylation
 Pronunciation link: No confirmed link found
 IPA: /ˈɑːksəˌdeɪtɪv ˌfɑːsfərɪˈleɪʃən/
 Phonetic Spelling: OK·suh·day·tiv foss·fer·uh·LAY·shuhn
22. Succinate
 Pronunciation link: <https://www.merriam-webster.com/dictionary/succinate>
 IPA: /ˈsʌksəˌneɪt/
 Phonetic Spelling: SUK·suh·nayt
23. Adenosine triphosphate
 Pronunciation link: <https://www.merriam-webster.com/dictionary/adenosine%20triphosphate>
 IPA: /əˈdenəˌsiːn traɪˈfəːsˌfeɪt/
 Phonetic Spelling: uh·DEN·uh·seen try·FOSS·fayt
24. Synthase
 Pronunciation link: <https://www.merriam-webster.com/dictionary/synthase>
 IPA: /ˈsɪnˌθeɪs/
 Phonetic Spelling: SIN·thays
25. Carbonyl
 Pronunciation link: <https://www.merriam-webster.com/dictionary/carbonyl>
 IPA: /ˈkɑːrbəˌnɪl/
 Phonetic Spelling: KAR·buh·nil
26. Cyanide
 Pronunciation link: <https://www.merriam-webster.com/dictionary/cyanide>
 IPA: /ˈsaɪəˌnaɪd/
 Phonetic Spelling: SY·uh·nyd
27. m-Chlorophenyl
 Pronunciation link: No confirmed link found
 IPA: /ˌmetə ˌklɔːrəʊˈfenəl/
 Phonetic Spelling: MET·uh klor·oh·FEN·uhl
28. Hydrazone
 Pronunciation link: No confirmed link found
 IPA: /ˈhaɪdrəˌzoʊn/
 Phonetic Spelling: HY·druh·zohn
29. Rotenone
 Pronunciation link: <https://www.merriam-webster.com/dictionary/rotenone>

- IPA: /'rootə,noun/
Phonetic Spelling: ROH·tuh·nohn
30. Antimycin
Pronunciation link: No confirmed link found
IPA: /,æntɪ'maɪsɪn/
Phonetic Spelling: an·tih·MY·sin
31. Ascorbate
Pronunciation link: <https://www.merriam-webster.com/dictionary/ascorbate>
IPA: /ə'skɔːr,bet/
Phonetic Spelling: uh·SKOR·bayt
32. N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride
Pronunciation link: No confirmed link found
IPA: /,tetrə'mɛθəl ,pærə ,fɛnələn'daɪəmiːn ,daɪhaɪdrə'klɔːraɪd/
Phonetic Spelling: tet·ruh·METH·uhl pair·uh fen·uh·leen·DY·uh·meen
dy·hy·druh·KLOR·yd
33. Biochemical
Pronunciation link: <https://www.merriam-webster.com/dictionary/biochemical>
IPA: / ,baɪoʊ'kɛmɪkəl/
Phonetic Spelling: by·oh·KEM·ih·kuhl
34. Bioenergetic
Pronunciation link: <https://www.merriam-webster.com/dictionary/bioenergetic>
IPA: / ,baɪoʊ ,ɛnər'dʒetɪk/
Phonetic Spelling: by·oh·en·er·JET·ik