Employee Discovery and Invention Report (EIR)

1. **Title of Discovery:**

   Stabilized coronavirus proteins for vaccination

2. **Brief Description of Discovery** *(in less than 200 words)*:

   Stabilized coronavirus proteins. This invention provides amino acid mutations to increase the stability of a prefusion conformation of coronavirus proteins including but not limited to: a) diisulfide linkages within or between protomers of the trimer, b) mutations between alpha helical regions to prevent formation of heptad repeats, c) cavity filling mutations, and d) mutations associated with functional changes in Spike, with the goal of improving immunogencity of coronavirus vaccines.

   Your discovery should be documented in your lab records. Please ensure that you maintain signed, witnessed, and dated lab records, but do not enclose them. E.g. see https://ttc.nci.nih.gov/pdfs/brochures/Keeping_Lab_Records.pdf

3 **Expanded Description of Discovery** *(Please attach a description of the technology in MS Word specifically describing what is new about the discovery including any sequences, compositions, structures, formulas, steps of a method etc. The description may be by reference to a separate document such as a copy of a report, preprint, manuscript or preliminary results and the like. Please include a MS Word copy if possible).* If this is a modification or improvement to an existing work or incorporates elements (software, confidential information, material) that are not original to you or your lab, please identify that work and any original creators.

3. **Technology Significance**

   Describe the unique advantages of this discovery over the current science/technology:

   Stabilized proteins will allow for improved immunogenicity of vaccines against betacoronaviruses, including MERS, SARS and HKU1

4. **Was this discovery made as part of a collaboration, contract, CRADA, or grant with an outside entity?**

(indicate yes or no)								
	x	Yes		No		Don't Know		
Is there a written agreement with the collaborator?	x	Yes		No		Don't Know		
Was the collaboration part of a CRADA?		Yes	x	No		Don't Know		
Did the collaborator provide materials?		Yes	x	No		Don't Know		
Did the discovery involve human materials or subjects?		Yes	x	No		Don't Know		

Name of Collaborator	**Material Provided (if any)**	**Agreement Type/ # (if known)**	**Date**
Jason McClellan, Dartmouth College; Andrew Ward, The Scripps Research Institute (through a 3 way RCA)			

5. **Publication or Other Disclosure of Discovery**

   Was the discovery: submitted to a journal, published, presented orally or as a poster, listed on a website, discussed with non-NIH personnel, or otherwise disclosed? If yes, describe (e.g. title, journal, public URL, hand-outs, location) and provide a date for each:

   The invention is based on the attached manuscript scheduled to be published by Feb 26 which describes the Spike trimer structure, but the invention comprises the Spike mutations that are informed by, but not included in the paper

   Are any future disclosures planned? If yes, provide estimated date(s):

   Provide 1) information/ attach PDFs regarding any disclosures above, and 2) citations to any work others have done in this specific area (e.g. scientific papers, patent /application numbers, public access web sites) and, if available, copies of cited documents:

   Citations:

6. **Technology Stage** *(Choose all that apply)*

   x Concept | x Prototype | Modification | *In vitro* | *In vivo* | Clinical | Final Product | Research Use

7. **Future Research Plans** Description of any additional research that is needed in order to complete development and testing of the invention (use an additional page if necessary):

   Generate *in vitro* and *in vivo* data on the constructs that have been designed

(a) Is this research presently being undertaken?	x	Yes		No	If yes, list any outside collaborator:	McLellan, Ward, and Denison
(b) Actively pursued by other PHS staff?		Yes	X		If yes, identify staff:	
(c) Actively undertaken by a corporate partner?		Yes	x	No	If yes, identify corporate partner:	
(d) Do you want to seek corporate partnership?	X	Yes		No		

   (e) Do you think this technology could form the basis of a "start-up" company? Yes | No | x Don't Know

9. **Commercial Potential** *(be creative)* Suggest any products, processes ,services you could envision resulting from this invention, and whether they can be developed in the near term (less than two years) or long term:

   Incorporated in optimized vaccines against coronaviruses

10. **Competition and Potential Users and Manufacturers**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

(a) Describe technologies, products, processes or services currently on the market of which you are aware that accomplish the purpose of this invention; or that are similar to this technology but used for a different purpose:	None known
(b) List any companies you believe may be interested in this technology:	Companies involved in development of vaccines
(c) If you have a contact at any of these companies, please provide name, email and phone numbers for each, if available:	

List the names and organizations of **all people** who participated in conceiving or continued development of the discovery/invention. Examples include those who made intellectual, theoretical, or innovative contribution to the discovery. In the case of software, those individuals who were involved in creating program code, manuals, flowcharts or any related items.

1. Submitting Contributor	Barney Graham	Organization:	VRC
2. Co-Contributor	Jason McLellan	Organization:	Dartmouth College
3. Co-Contributor	Andrew Ward	Organization:	The Scripps Research Institute
4. Co-Contributor	Robert Kirchdoerfer	Organization:	The Scripps Research Institute

Enter additional Co-Contributor's names and organizations as necessary:

5. Christopher A. Cottrell (The Scripps Research Institute)
6. Nianshuang Wang (Dartmouth College)
7. Jesper Pallesen (The Scripps Research Institute)
8. Hadi M. Yassine (NIH during invention, currently U of Qatar)
9. Hannah L. Turner (The Scripps Research Institute)
10. Kizzmekia S. Corbett (NIH)

An Additional Contributor Information document (to follow) is to be completed for each contributor listed above. If required, extra forms may be downloaded at: http://www.ott.nih.gov/sites/default/files/documents/docs/eir-additional-contributor.docx

In addition to the above names, identify any individuals who could merit authorship credit of any associated publication:

Masaru Kanekiyo, Gordon Joyce, Mark Denison (Vanderbilt University)

NOTICE: There may be fewer individuals listed as contributors than named as coauthors. *Please be aware that inventorship is strictly defined in patent law. Accordingly, contributors you list in this section will be named on patent applications resulting from this EIR only if their contributions meet this legal standard.* A co-author may or may not qualify based on the particular facts; if you have any questions, contact your TDC.

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an **obligation to report** and assign inventions to the United States of America as represented by the Government of the United States (the Department of Health and Human Services). Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by HHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your TDC and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

Detailed Contributor Information begins on next page

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 1

Name

	Barney	♦Middle	Scott		Graham	Suffix	
Degree	MD, PhD	♦Citizenship	(b) (6)				
♦Employee Identification No.	001-0633-078			♦Associated project NIH Z01 Project Number(s)			

♦ **Describe this individual's contribution to the discovery.**

Conceived of the strategy for designing mutations to stabilize proteins

Current Organization Information:

♦Organization Name	NIAID, NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Deputy Director						
♦Office Address	2502 Building 40						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX	(b) (6)	Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	US
Phone		Email					

Please identify with a "X" If this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundationo		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

(b) (6) understand the information submitted in the EIR.

Signature

3-10-2016
Date

3 of 10 (VA 7/8/16

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 2

Name

♦First | ♦Middle | ♦Last | Suffix

Degree | ♦Citizenship

♦HHS ID # (e.g. 999-9999-999) | ♦Associated project NIH Z01 Project #

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name

Division/Branch/Laboratory

♦Title

♦Office Address

♦City | ♦State | ♦Zip | ♦Country

♦Email | Telephone | Fax | Other contact# (optl)

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street | ♦City | ♦State | ♦Zip code

♦ Country | Phone | ♦Email

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

VA 7/8/2016

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 3

Name

♦First | ♦Middle | ♦Last | Suffix

Degree | ♦Citizenship

♦HHS ID # (e.g. 999-9999-999) | ♦Associated project NIH Z01 Project #

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name

Division/Branch/Laboratory

♦Title

♦Office Address

♦City | ♦State | ♦Zip | ♦Country

♦Email | Telephone | Fax | Other contact# (optl)

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street | ♦City | ♦State | ♦Zip code

♦ Country | Phone | ♦Email

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

CRADA Personnel	Howard Hughes Fellow	ORISE Fellow	NIH-ORAU
Clinical Fellow	Gates Foundation	NRSA Fellowship	Visiting Fellowship
Fogarty Scholar	IRTA Fellowship Program	Postdoctoral Fellow	Other (specify below)*
Oxford-Cambridge Scholars Program	National Research Council Award	Research Fellowship	NIH Contract Employee – specify employer name *
CNRM Personnel (HJF)	Society Fellows specify below	Graduate Partnership Program	

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

VP 7/8/2016

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 4

Name

♦First		♦Middle		♦Last		Suffix	
Degree		♦Citizenship					
♦HHS ID # (e.g. 999-9999-999)				♦Associated project NIH Z01 Project #			

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title							
♦Office Address							
♦City		♦State		♦Zip		♦Country	
♦Email		Telephone		Fax		Other contact# (optl)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street		♦City		♦State		♦Zip code	
♦Country		Phone		♦Email			

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

VA 7/8/2016

Employee Discovery and Invention Report (EIR)
Information

♦ Indicates a required field

Information about this Form

Reporting an invention is required as part of your Government service, and supports the mission of your IC and the NIH in advancing public health. An EIR should be completed for each discovery or invention* that is:

a) An innovation;
b) A new or improved method or process;
c) Believed to have potential commercial value (e.g. a new reagent, unique antibody, vaccine, medical device, or therapeutic compound); **or**
d) Requested from a commercial organization for use or resale.

If you are employed by HHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

IC means a Public Health Service (PHS) Contributor's Institute, Center, or Office (includes NIH, FDA and CDC).

COMPLETION OF THE EIR

1. Complete the form by filling in the shaded fields. For "check boxes" insert "X";
2. Once completed, have each contributor sign their Contributor Information Sheet;
3. Questions regarding the completion of the EIR should be referred to your TDC;
4. Email the completed electronic EIR template and any related documents to your TDC; and
5. After review by your TDC, email a signed copy of the final EIR to your TDC.
6. The TDC will then forward the completed and signed EIR to the Office of Technology Transfer (OTT). If your IC in decides not to file a patent application on your invention you may contact your TDC to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

General questions regarding the form may be directed to your TDC or the NIH Office of Technology Transfer (OTT). It is suggested, particularly if you leave government service and are receiving royalties, that you keep the Office of Financial Management apprised of changes in your official address.

Thank you for your contribution toward improving public health!

Privacy Act Notice: HHS is collecting this information under authority of 45 CFR Part 7 "Employee Inventions". The information will be maintained as a part of the System of Records: 09-25-0168, "Invention, Patent and Licensing Documents." Provision of this information is mandatory and will be used as the initial step toward obtaining patent protection of inventions submitted by HHS employees, granting licenses to HHS inventions, administering and providing royalty payments to HHS inventors, and the intended "routine uses" of the information. Failure to provide complete information may adversely affect the Government's rights to future patent applications and licensing agreements.

***What is the difference between a discovery and an invention?**

Discovery

- Elucidating something that already exists
- Embodied in Nature
- Discovery involves describing something
- Product of Nature
- e.g. A botanist discovers a new plant species on an island

Invention

- An innovation that did not previously exist
- Embodied in human-made artifact
- Always involves creating something
- Produced through human thought
- A botanist invents a new topical antibiotic formulation using the plant oils

Employee Discovery and Invention Report (EIR)
Administration Section
(To be completed by the IC Technology Transfer Office)

♦ Indicates a required field

Lead IC sponsoring this invention:	NIAID	Division/Lab/ Branch	VRC
Identify any other ICs (if necessary, include Division/Lab/Branch	N/A		
Confirm the scientist who will serve as the key point of contact (Scientific Point of Contact or SPC) to receive and respond to patent correspondence. This may be different from the person identified as the Submitting Contributor.			Graham

IC CRADA Determination: (Answer required if the EIR is related to a CRADA)

	The invention was conceived or first actually reduced to practice in the performance of the activities under the CRADA Research Plan during the term of the CRADA. Enter Y or N.

IC is requesting the following action regarding this EIR. *Only 1 of the following option boxes should be checked.*

Do Not File

	Do not file; EIR is being submitted due to a request for material(s) licensing. Requester's contact information is attached
	Do not file; market as a research material
	Do not file a patent application based on other patent and/or policy reasons

Evaluation/Searching

	Request evaluation and recommendation, Outside search/opinion are authorized, if needed
	Request evaluation and recommendation, Outside search/opinion is not authorized

Patent Application Filing

x	Emergency Patent Filing - File immediate provisional patent application based on an imminent publication. Authorization for filing is provided. In the "Additional Information or Instructions" box enter the date, location of public release and any other instructions
	File Patent: File a fully enabled provisional patent application
	File Patent/Patent Opinion: File a fully enabled provisional patent application and conduct a patentability opinion/assessment immediately after the provisional patent application has been filed
	Patent Opinion/File Patent: Conduct a patentability opinion/assessment (Completed by contract law firm). If the patentability opinion/assessment is positive, proceed with the filing a fully enabled provisional patent application
	Third Party Filing Patent Lead: Third party has already filed or will be doing the filing. Please provide 3rd Party contact information. Application number(s), filing date(s), and copy(ies) of application(s) should be provided as well as filing receipts or paperwork identifying the inventors as filed
	Other Filing Instructions

Additional Information or Instructions

Is the IC submitting the EIR represented by a Service Center?	No
If yes, identify IC:	N/A

All patent recommendations and patent correspondence will be directed the Lead IC's central email account.

Name of LPM designated for this EIR by IC:

The TDC, IC delegate, or Service Center Representative confirms receiving the EIR and acknowledges a complete EIR packet is being forwarded to OTT.

Name	Vincent Feliccia, JD, PhD	Date	7/8/2016
Title	Branch Chief, TTIPO, NIAID		
Signature	(b) (6)		

Authorized IC official for expenditure of IC funds for patent related expenses or attach authorization memo.

Name	Michael Mowatt, PhD	Date	8 JUL 2016
Title	NIAID TDC and Director, TTIPO, NIAID		
Signature	(b) (6)		

An OCR'd PDF containing the signed EIR containing all documents such as manuscripts, presentations, articles and citations referred to in the EIR, as well as any related IC reviews and authorization documents should be forwarded to OTT at ottfileroom@mail.nih.gov. The email's subject line should include "New EIR for IC, (PI's Last Name), (IC Ref. #, if exists)." Original formatted documents, i.e. MS Word, PowerPoint, should be attached for EIRs with recommendations of "Evaluate", "File Patent", "Patent Opinion" or any combination of these.

Employee Discovery and Invention Report (EIR)
Appendix A - Supplemental Information Sheet for Research Materials

This attachment requests information regarding materials that may be available for potential licensing. The **Set 1** questions elicit general information regarding specific materials required to practice the invention or ancillary materials created during the course of development that may be potentially licensed. The **Set 2** questions SHOULD BE ANSWERED IF THIS EIR is being submitted based on an outside party's request for licensing a material.

Set 1. General material information. *(Not required if Set 2 Questions are completed)*

a) Identify those materials made during the course of research that are specifically required to practice this invention. Please identify each unique material or chemical compound developed that are related to this EIR.

b) Identify those material(s) made during the course of research that may be available for licensing as a research material. Please identify each unique material or chemical compound developed that are related to this EIR.

c) Material citation or other source:

d) Has material been deposited in the ATCC or similar repository? If yes, please provide the repository and catalog reference number.

e) Were any of the materials necessary to use or make the Material acquired from someone outside NIH? If yes and not already listed in Question 5, please provide contact information and a copy of any document that records this transfer.

Set 2. If a prospective licensee is interested in a material answer the following questions.

Provide a summary regarding the approximate difficulty/time/cost/effort for your laboratory to provide the material to an outside for-profit requestor. Is it a limited resource?

Identify and describe the Material Type:

Examples of potential material types:

Antibodies: monoclonal, polyclonal
Cell Lines: uninfected cells, infected cells, hybridomas
DNA/RNA: genetic clones, expression vectors
PCR reagents
Proteins/Peptides
Purified Proteins
Viruses: virus isolates, drug resistant, virus isolates, recombinant vaccinia
Opportunistic Infections: (e.g. Candida, Cryptococcus, cryptosporidium, cytomegalovirus, mycobacterium, mycoplasma, pneumocystis, toxoplasma)
Model Organisms (e.g. strain, species)
Chemical Compounds

Designation: (Laboratory nomenclature)

Source of Material: (i.e. human, mouse, rat)

Reference Citation or other source:

Has the material been deposited in the ATCC or similar repository? If Yes, provide repository & catalog. ref.#s.

How can it be provided: (i.e. 2ml vial of frozen cells, plasmid)

Current quantities available for distribution: (i.e. 10 vials)

Recommended propagation medium & growth characteristics: (i.e. expression level, titer, temp., passages)

Recommended freeze medium:

Sterility: (i.e. negative for bacteria, fungi and mycoplasma)

Morphology: (i.e. epithelial-like, lymphoblast-like, fibroblast-like)

Recommended Storage: (i.e. liquid nitrogen)

Employee Discovery and Invention Report (EIR)
Appendix B - Supplemental Information Sheet for Software

The purpose of this attachment is to provide information regarding evaluation of software for potential licensing. The following questions should be answered as completely as possible.

1. Does this software contain code obtained from a third-party or covered by any Open Source License (e.g., collaborator, under a software agreement, a vendor, purchased etc.)? If Yes, please explain.

2. Has this software been previously copyrighted? If yes, by whom?

3. Did you use outsiders to beta-test code? If yes, was this done under an agreement?

4. How would the lab generally classify the use of this software (e.g., imaging, array analysis, mapping etc.)?

5. From the lab's perspective, what would be the preferred way of distributing this software?

6. What are the operating system requirements to run this software?

7. In which computer language is the software code written?

8. What stage of development is the software? Select one of the following:

   - ☐ Ready to use by anyone
   - ☐ Useable with some effort or assistance
   - ☐ Needs substantial further development

9. Is the lab willing to release the source code?

10. Is the lab willing to prepare a demonstration version of the software to give to prospective licensees?

11. Were the current or prior versions distributed? If yes, explain and supply date of distribution and any distribution agreement (if any).

12. Was a government contractor involved in the writing or development of any of the code? If yes, identify the individual.

Construct Name	Concept	Designer	Design Date
MERS.C6.NW0	Control - Wild Type	McLellan	7-1-2015
MERS.C6.NW1	mutate S2' Furin site	McLellan	1-11-2015
MERS.C6.NW2	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW3	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW4	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW5	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW6	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW7	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW8	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW9	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015
MERS.C6.NW10	Proline substitution	McLellan	1-11-2015
MERS.C6.NW11	Proline substitution	McLellan	1-11-2015
MERS.C6.NW12	Proline substitution	McLellan	1-11-2015
MERS.C6.NW13	Proline substitution	McLellan	2-9-2015
MERS.C6.NW14	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW15	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW16	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW17	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW18	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW19	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW20	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW21	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW22	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW23	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW24	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW25	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015
MERS.C6.NW26	CAV filling	McLellan	2-9-2015
MERS.C6.NW27	CAV filling	McLellan	2-9-2015
MERS.C6.NW28	Repacking (intra subunit)	Kanekiyo	2-9-2016
MERS.C6.NW29	Repacking (intra subunit)	Kanekiyo	2-9-2016
MERS.C6.NW30	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW31	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW32	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW33	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW34	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW35	Repacking (intra subunit)	Kanekiyo	2-9-2016
MERS.C6.NW36	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW37	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW38	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW39	Repacking (RBD base stabilization)	Kanekiyo	2-9-2016
MERS.C6.NW40	Hydrophilic residue at bottom of central water cavit	Ward Lab	3/10/2016
MERS.C6.NW41	improve hydrophobic packing of central helix	Ward Lab	3/10/2016
MERS.C6.NW42	improve hydrophobic packing of central helix	Ward Lab	3/10/2016
MERS.C6.NW43	improve hydrophobic packing at base of S2	Ward Lab	3/10/2016
MERS.C6.NW44	S1-S1 disulfide crosslink	Ward Lab	3/10/2016
MERS.C6.NW45	S1-S1 disulfide crosslink	Ward Lab	3/10/2016

MERS.C6.NW46	S1-S2 disulfide crosslink	Ward Lab	3/10/2016
MERS.C6.NW47	S1-S2 disulfide crosslink	Ward Lab	3/10/2016
MERS.C6.NW48	hydrophobic pocket filling	Ward Lab	3/10/2016
MERS.C6.NW49	S1-S2 disulfide crosslink	Corbett	2/29/2016
MERS.C6.NW50	S1-S2 disulfide crosslink	Corbett	2/29/2016
MERS.C6.NW51	S1-S2 disulfide crosslink	Corbett	2/29/2016
MERS.C6.NW52	Proline substitution	Corbett	2/29/2016
MERS.C6.NW53	Proline substitution	Corbett	2/29/2016
MERS.C6.NW54	Proline substitution	Corbett	2/29/2016
MERS.C6.NW55	Proline substitution	Corbett	2/29/2016
MERS.C6.NW56	Proline substitution	Corbett	2/29/2016
MERS.C6.NW57	Proline substitution	Corbett	2/29/2016
MERS.C6.NW58	Proline substitution	Corbett	2/29/2016
MERS.C6.NW59	Proline substitution	Corbett	2/29/2016
MERS.C6.NW60	Proline substitution	Corbett	2/29/2016
MERS.C6.NW61	Proline substitution	Corbett	2/29/2016
MERS.C6.NW62	Proline substitution	Corbett	2/29/2016
MERS.C6.NW63	Proline substitution	Corbett	2/29/2016
MERS.C6.NW64	Proline substitution	Corbett	2/29/2016
MERS.C6.NW65	Proline substitution	Corbett	2/29/2016
MERS.C6.NW66	Proline substitution	Corbett	2/29/2016
MERS.C6.NW67	Proline substitution	Corbett	2/29/2016
MERS.C6.NW68	Proline substitution	Corbett	2/29/2016

Mutations

None (1-1291, S1/S2 furin site mutated,Foldon-3C-His-Strep)
884-RSAR-887 to 884-ASAG-887
884-RSAR-887 to 884-ASAG-887, L1058P
884-RSAR-887 to 884-ASAG-887, D1059P
884-RSAR-887 to 884-ASAG-887, V1060P
884-RSAR-887 to 884-ASAG-887, L1061P
884-RSAR-887 to 884-ASAG-887, F1044P
884-RSAR-887 to 884-ASAG-887, I1047P
884-RSAR-887 to 884-ASAG-887, A1049P
884-RSAR-887 to 884-ASAG-887, T1014P
D1059P
V1060P
L1061P
V1060P,L1061P
N1072F,A1083I
N1072F,L1086F
N1072F,V1087I
N1072F,E1090I
T1076F,A1083I
T1076F,L1086F
T1076F,V1087I
T1076F,E1090I
T1076I,A1083I
T1076I,L1086F
T1076I,V1087I
T1076I,E1090I
A1018V
A1018I
E793M, K1102F
E793M, K1102F, H1138F
D1068M, R1069W
A1083L
A1083L, V1087I
A1083L, V1087I, E1090L
A834L, Q1084M
Q1066M
S454F
R921W
S612F, G1052F
P476V, T477A, R1057W
A1083S
E1090I
Q1097I
D1101F
T63C, V631C
T63C, Q638C

Q733C, D940C
S676C, D910C
A653W
V1087C
A432C, L1058C
A432C, D1059C
S919P
A920P
A968P
A969P
I970P
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T803P
V804P

Sequence

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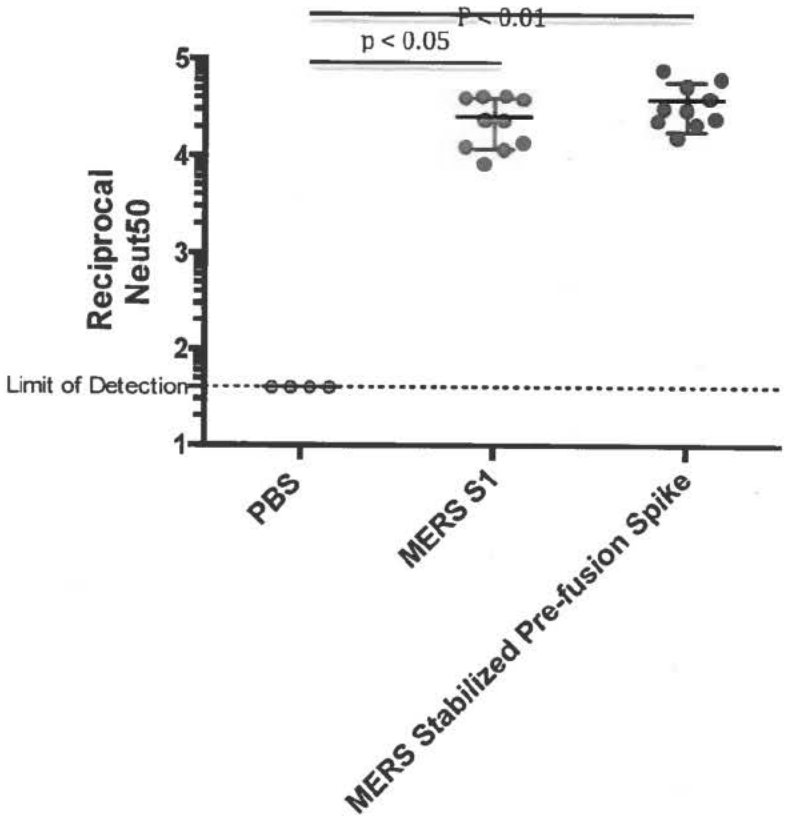

Figure 1. Neutralizing Antibodies in Mice Immunized with MERS stabilized pre-fusion spike. Mice were immunized with 10 μg of mock/PBS (gray) (N=4), MERS S1 protein (N=10), or MERS stabilize pre-fusion spike protein (N=10) intramuscularly with Sigma Adjuvant System at weeks 0 and 3. Two weeks following last immunization, serum was collected and testing for neutralizing antibodies by MERS pseudo-neutralization assay. Serum was diluted, in triplicate, and incubated with MERS pseudovirus prior to inoculation of Huh7.5 cells. Dilution curves were fitted to mock cells and cells exposed to un-neutralized virus as 100% and 0% neutralization, respectively. Neut50 was calculated as the dilution of serum needed to neutralize 50% of MERS pseudovirus. Each dots represents reciprocal neut50 titer of one mouse. Dotted line indicates the limit of detection of the assay (1:40 neut50). Statistics were calculated using One-way ANOVA tests with Kruskal-Wallis multiple comparison test. (GraphPad Prism 6).

Table 1. Conception of MERS Pre-fusion Stabilizing Spike Mutations

Construct Name	Concept	Designer	Design Date	Mutations
MERS.C6. NW0	Wild-type	McLellan	7-1-2015	None (1-1291, S1/S2 furin site mutated, Foldon-3C-His-Strep)
MERS.C6. NW1	mutate S2' Furin site	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887
MERS.C6. NW2	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, L1058P
MERS.C6. NW3	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, D1059P
MERS.C6. NW4	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, V1060P
MERS.C6. NW5	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, L1061P
MERS.C6. NW6	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, F1044P
MERS.C6. NW7	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, I1047P
MERS.C6. NW8	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, A1049P
MERS.C6. NW9	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, T1014P
MERS.C6. NW10	Proline substitution	McLellan	1-11-2015	D1059P
MERS.C6. NW11	Proline substitution	McLellan	1-11-2015	V1060P
MERS.C6. NW12	Proline substitution	McLellan	1-11-2015	L1061P
MERS.C6. NW13	Proline substitution	McLellan	2-9-2015	V1060P,L1061P
MERS.C6. NW14	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,A1083I
MERS.C6. NW15	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,L1086F
MERS.C6. NW16	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,V1087I
MERS.C6. NW17	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,E1090I
MERS.C6. NW18	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,A1083I
MERS.C6. NW19	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,L1086F
MERS.C6. NW20	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,V1087I
MERS.C6. NW21	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,E1090I
MERS.C6. NW22	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,A1083I
MERS.C6. NW23	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,L1086F
MERS.C6. NW24	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,V1087I
MERS.C6. NW25	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,E1090I
MERS.C6. NW26	CAV filling	McLellan	2-9-2015	A1018V
MERS.C6. NW27	CAV filling	McLellan	2-9-2015	A1018I
MERS.C6. NW28	Repacking (intra subunit)	Kanekiyo	2-9-2016	E793M, K1102F
MERS.C6. NW29	Repacking (intra subunit)	Kanekiyo	2-9-2016	E793M, K1102F, H1138F
MERS.C6. NW30	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016	D1068M, R1069W
MERS.C6. NW31	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L
MERS.C6. NW32	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L, V1087I
MERS.C6. NW33	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L, V1087I, E1090L
MERS.C6. NW34	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016	A834L, Q1084M

MERS.C6.NW35	Repacking (intra subunit)	Kanekiyo	2-9-2016	Q1066M
MERS.C6.NW36	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	S454F
MERS.C6.NW37	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016	R921W
MERS.C6.NW38	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016	S612F, G1052F
MERS.C6.NW39	Repacking (RBD base stabilization)	Kanekiyo	2-9-2016	P476V, T477A, R1057W
MERS.C6.NW40	Hydrophilic residue at bottom of central water cavity	Ward	3/10/16	A1083S
MERS.C6.NW41	improve hydrophobic packing of central helix	Ward	3/10/16	E1090I
MERS.C6.NW42	improve hydrophobic packing of central helix	Ward	3/10/16	Q1097I
MERS.C6.NW43	improve hydrophobic packing at base of S2	Ward	3/10/16	D1101F
MERS.C6.NW44	S1-S1 disulfide crosslink	Ward	3/10/16	T63C, V631C
MERS.C6.NW45	S1-S1 disulfide crosslink	Ward	3/10/16	T63C, Q638C
MERS.C6.NW46	S1-S2 disulfide crosslink	Ward	3/10/16	Q733C, D940C
MERS.C6.NW47	S1-S2 disulfide crosslink	Ward	3/10/16	S676C, D910C
MERS.C6.NW48	hydrophobic pocket filling	Ward	3/10/16	A653W
MERS.C6.NW49	S1-S2 disulfide crosslink	Corbett	2/29/16	V1087C
MERS.C6.NW50	S1-S2 disulfide crosslink	Corbett	2/29/16	A432C, L1058C
MERS.C6.NW51	S1-S2 disulfide crosslink	Corbett	2/29/16	A432C, D1059C
MERS.C6.NW52	Proline substitution	Corbett	2/29/16	S919P
MERS.C6.NW53	Proline substitution	Corbett	2/29/16	A920P
MERS.C6.NW54	Proline substitution	Corbett	2/29/16	A968P
MERS.C6.NW55	Proline substitution	Corbett	2/29/16	A969P
MERS.C6.NW56	Proline substitution	Corbett	2/29/16	I970P
MERS.C6.NW57	Proline substitution	Corbett	2/29/16	F972P
MERS.C6.NW58	Proline substitution	Corbett	2/29/16	A973P
MERS.C6.NW59	Proline substitution	Corbett	2/29/16	N1042P
MERS.C6.NW60	Proline substitution	Corbett	2/29/16	T1043P
MERS.C6.NW61	Proline substitution	Corbett	2/29/16	F1044P
MERS.C6.NW62	Proline substitution	Corbett	2/29/16	G1045P
MERS.C6.NW63	Proline substitution	Corbett	2/29/16	A1045P
MERS.C6.NW64	Proline substitution	Corbett	2/29/16	I1046P
MERS.C6.NW65	Proline substitution	Corbett	2/29/16	K801P
MERS.C6.NW66	Proline substitution	Corbett	2/29/16	V802P
MERS.C6.NW67	Proline substitution	Corbett	2/29/16	T803P
MERS.C6.NW68	Proline substitution	Corbett	2/29/16	V804P

Stabilizing mutations were added to MERS England strain spike protein (MERS.C6.NW0) to stabilize the protein in pre-fusion form. Preliminary studies reveal mutations V1060P and L1061P (yellow) increase the stability of MERS spike trimer, but additional stabilizing mutations are needed. Additional mutations,

contributed by the Graham lab (blue), McLellan lab (black) and Ward (lab), will be tested in the future. Sequences are attached in excel format.

Data figures and Description

Structure-based stabilization of beta-CoV prefusion trimers

Stabilization of prefusion proteins is a method often used to produce highly immunogenic protein subunit vaccines [3, 4]. Details uncovered in the HKU1-CoV prefusion S structure provided us with indications as to how we could stabilize other beta-CoV spike trimers in the prefusion conformation. We tested various stabilizing mutations (**table 1, figure 1a**). Notably, mutations V1060P (top 3) and L1061P (top 4), in MERS-CoV spike stabilized MERS-CoV S in prefusion confirmation **(figure 1)**; namely this construct is called MERS.C6.NW13 **(table 1)**. Use of proline substitution to stabilize helices is a method previously utilized to stabilize prefusion RSV-F [5]. We hypothesize that mutations V1060P and L1061P, located at top of MERS S2 central helix and HR1, prevent pre-to-postfusion conformational changes. Prefusion stabilization is preliminarily indicated by increased protein expression levels of MERS.C6.NW13 when compared to expression levels of wild-type MERS-CoV spike (MERS.C6.NW0 on **table 1**) (**figure 1b,c**). WT MERS-CoV S likely spontaneously flips from pre-to-postfusion conformation, thus resulting in expression levels that are nearly undetectable. As well, corresponding mutations in SARS-CoV and HKU1-CoV spike backbones increased expression above wild-type protein (data not shown). These data serve as foundations for structure-based design of beta-CoV vaccine immunogens.

Advancing towards a general solution for beta-CoV vaccine design

While HKU1-CoV infection typically manifests as asymptomatic or mild respiratory disease [6], it is closely related to SARS-CoV and MERS-CoV, which are both responsible for previous deadly outbreaks [7]. The necessity for a general vaccine solution for human beta-Coronaviruses has become more apparent following recent MERS-CoV outbreaks [8]. Beta-CoVs thrive in animal reservoirs and are constantly poised for emergence into humans. This phenomenon was most recently suggested with bat SARS-like CoV, WIV1, which is thought to be an imminent human health threat [9]. To that end, we plan to test stabilized prefusion CoV spike trimers as vaccine candidates. To date, we have vaccinated mice with stabilized MERS spike protein; we used MERS S1 protein as a means for comparison, as it is known to induce robust neutralizing antibody responses [10]. Preliminary findings suggest vaccination with MERS S1 and stabilized prefusion MERS spike induce similar robust levels of neutralizing antibodies against homologous MERS Eng pseudovirus (**figure 2a**) and heterologous MERS Korea002 pseudovirus (**figure 2b**). However, vaccination with stabilized prefusion MERS spike induces significantly more neutralizing antibodies against genetically-distant heterologous MERS strain Florida-USA2 than MERS S1 vaccination (**figure 2c**). These findings suggest inclusion of S2 domain in MERS-CoV vaccine may increase capacity of the vaccine to elicit broadly neutralizing antibodies. Moving forward, we will dissect the epitope specificities of antibodies from these mice. Additionally, it will be interesting to prime with MERS stabilized prefusion spike DNA and boost with protein, as we previously found MERS S1 DNA vaccine protects against MERS challenge [10]. We also plan to use multiple beta-CoV prefusion spike trimers in combination vaccine regimens to assess induction of cross-reactive beta-CoV antibodies and cross-protection. As the ultimate goal of this AIM is to accelerate towards a universal beta-CoV vaccine that would be effective in the onset of an emerging CoV pandemic, it will prove

beneficial to test prefusion CoV spike protein vaccine platforms in the context of beta-CoVs that are particularly hypothesized to be on the cusp of human emergence.

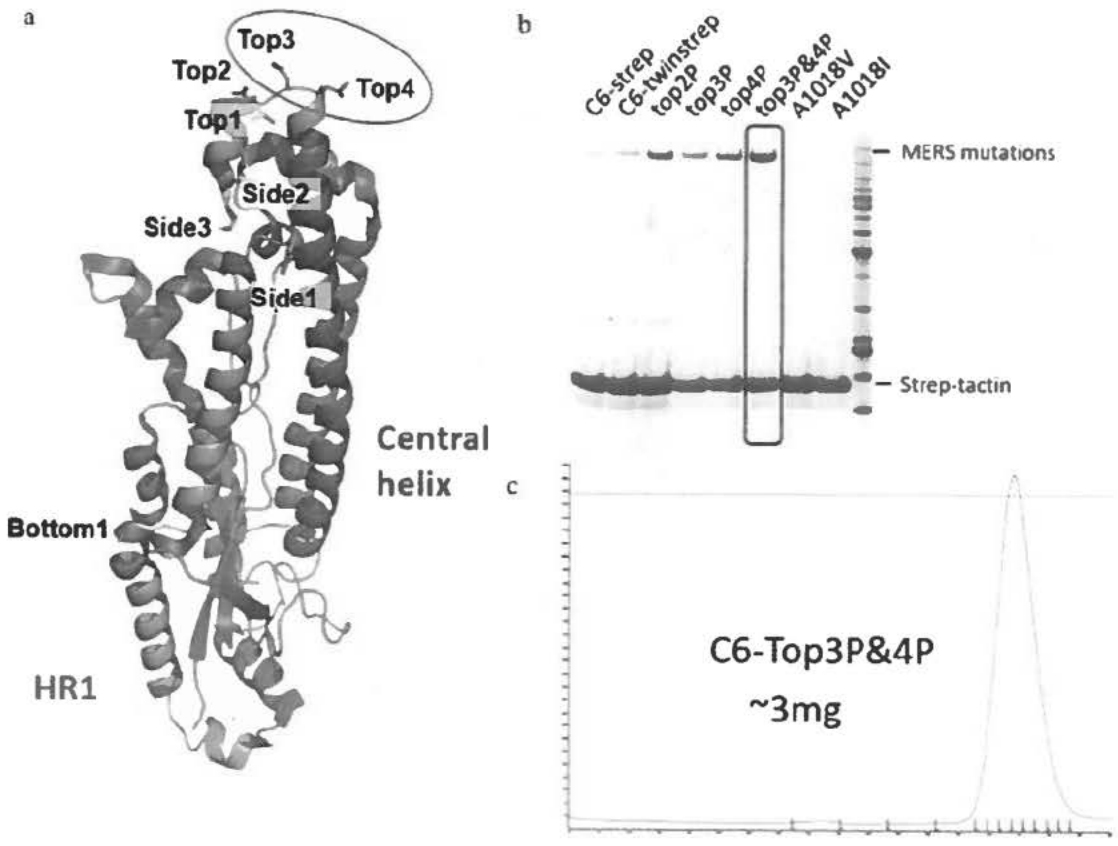

Figure 1. Stabilization of MERS-CoV using mutations V1060P (top 3) and L1061P (top 4). (a) Location of various stabilization design conceptions, corresponding to table 1. V1060P (top 3) and L1061P (top 4) (red circle) are located at the top of S2 HR1 and the S2 central helix. V1060P (top 3) and L1061P (top 4) mutations were expressed individually and in combination and purified. Protein expression levels and purity were determined by **(b)** gel electrophoresis and **(c)** size exclusion chromatography.

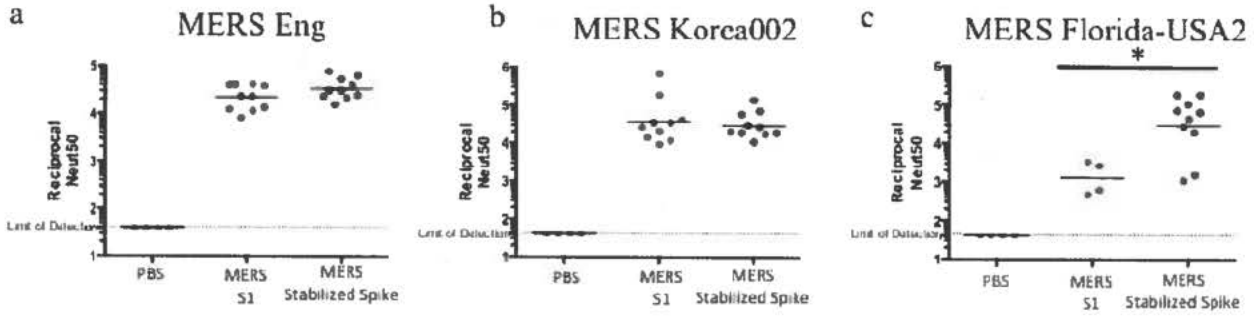

Figure 2. Neutralizing antibodies in mice immunized with MERS stabilized pre-fusion spike. Mice were immunized with 10 μg of mock/PBS (gray) (N=4), MERS S1 protein (red) (N=10), or MERS stabilized prefusion spike protein (blue) (N=10) intramuscularly with Sigma Adjuvant System at weeks 0 and 3. Two weeks following the final immunization, serum was collected and testing for neutralizing antibodies by MERS pseudo-neutralization assay. Serum was diluted, in triplicate, and incubated with MERS **(a)** Eng, **(b)** Korea002, and **(c)** Florida-USA2 pseudovirus prior to inoculation of Huh7.5 cells. Dilution curves were fitted to mock cells and cells exposed to un-neutralized virus as 100% and 0% neutralization, respectively. Neut50 titers were calculated as the dilution of serum needed to neutralize 50% of MERS pseudovirus. Each dots represents reciprocal neut50 titer of one mouse. Dotted line indicates the limit of detection of the assay (1:40 neut50). A solid line represents each group's geometric mean neut50 titer. Statistics were calculated using unpaired t-tests (GraphPad Prism 6). P-value < 0.05 = *.

Table 1. Conception of MERS Pre-fusion Stabilizing Spike Mutations

Construct Name	Concept	Designer	Design Date	Mutations
MERS.C6.NW0	Wild-type (1-1291, S1/S2 furin site mutated, Foldon-3C-His-Strep)	McLellan	7-1-2015	None
MERS.C6.NW1	mutate S2' Furin site	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887
MERS.C6.NW2	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, L1058P
MERS.C6.NW3	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, D1059P
MERS.C6.NW4	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, V1060P
MERS.C6.NW5	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, L1061P
MERS.C6.NW6	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, F1044P
MERS.C6.NW7	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, I1047P
MERS.C6.NW8	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, A1049P
MERS.C6.NW9	mutate S2' Furin site,Proline substitution	McLellan	1-11-2015	884-RSAR-887 to 884-ASAG-887, T1014P
MERS.C6.NW10	Proline substitution	McLellan	1-11-2015	D1059P
MERS.C6.NW11	Proline substitution	McLellan	1-11-2015	V1060P
MERS.C6.NW12	Proline substitution	McLellan	1-11-2015	L1061P
MERS.C6.NW13	Proline substitution	McLellan	2-9-2015	V1060P,L1061P
MERS.C6.NW14	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,A1083I
MERS.C6.NW15	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,L1086F
MERS.C6.NW16	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,V1087I
MERS.C6.NW17	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	N1072F,E1090I
MERS.C6.NW18	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,A1083I
MERS.C6.NW19	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,L1086F
MERS.C6.NW20	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,V1087I
MERS.C6.NW21	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076F,E1090I
MERS.C6.NW22	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,A1083I
MERS.C6.NW23	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,L1086F
MERS.C6.NW24	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,V1087I
MERS.C6.NW25	CAV filling (for central helix, stablize trimer)	McLellan	2-9-2015	T1076I,E1090I
MERS.C6.NW26	CAV filling	McLellan	2-9-2015	A1018V
MERS.C6.NW27	CAV filling	McLellan	2-9-2015	A1018I
MERS.C6.NW28	Repacking (intra subunit)	Kanekiyo	2-9-2016	E793M, K1102F
MERS.C6.NW29	Repacking (intra subunit)	Kanekiyo	2-9-2016	E793M, K1102F, H1138F
MERS.C6.NW30	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016	D1068M, R1069W
MERS.C6.NW31	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L

MERS.C6.NW32	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L, V1087I
MERS.C6.NW33	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	A1083L, V1087I, E1090L
MERS.C6.NW34	Repacking (inter subunit, dimer stabilization)	Kanekiyo	2-9-2016	A834L, Q1084M
MERS.C6.NW35	Repacking (intra subunit)	Kanekiyo	2-9-2016	Q1066M
MERS.C6.NW36	Repacking (inter subunit, trimer stabilization)	Kanekiyo	2-9-2016	S454F
MERS.C6.NW37	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016	R921W
MERS.C6.NW38	Repacking (subdomain stabilization)	Kanekiyo	2-9-2016	S612F, G1052F
MERS.C6.NW39	Repacking (RBD base stabilization)	Kanekiyo	2-9-2016	P476V, T477A, R1057W
MERS.C6.NW40	Hydrophilic residue at bottom of central water cavity	Ward	3/10/16	A1083S
MERS.C6.NW41	improve hydrophobic packing of central helix	Ward	3/10/16	E1090I
MERS.C6.NW42	improve hydrophobic packing of central helix	Ward	3/10/16	Q1097I
MERS.C6.NW43	improve hydrophobic packing at base of S2	Ward	3/10/16	D1101F
MERS.C6.NW44	S1-S1 disulfide crosslink	Ward	3/10/16	T63C, V631C
MERS.C6.NW45	S1-S1 disulfide crosslink	Ward	3/10/16	T63C, Q638C
MERS.C6.NW46	S1-S2 disulfide crosslink	Ward	3/10/16	Q733C, D940C
MERS.C6.NW47	S1-S2 disulfide crosslink	Ward	3/10/16	S676C, D910C
MERS.C6.NW48	hydrophobic pocket filling	Ward	3/10/16	A653W
MERS.C6.NW49	S1-S2 disulfide crosslink	Corbett	2/29/16	V1087C
MERS.C6.NW50	S1-S2 disulfide crosslink	Corbett	2/29/16	A432C, L1058C
MERS.C6.NW51	S1-S2 disulfide crosslink	Corbett	2/29/16	A432C, D1059C
MERS.C6.NW52	Proline substitution	Corbett	2/29/16	S919P
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MERS.C6.NW54	Proline substitution	Corbett	2/29/16	A968P
MERS.C6.NW55	Proline substitution	Corbett	2/29/16	A969P
MERS.C6.NW56	Proline substitution	Corbett	2/29/16	I970P
MERS.C6.NW57	Proline substitution	Corbett	2/29/16	F972P
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MERS.C6.NW62	Proline substitution	Corbett	2/29/16	G1045P
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MERS.C6.NW64	Proline substitution	Corbett	2/29/16	I1046P
MERS.C6.NW65	Proline substitution	Corbett	2/29/16	K801P

MERS.C6.NW66	Proline substitution	Corbett	2/29/16	V802P
MERS.C6.NW67	Proline substitution	Corbett	2/29/16	T803P
MERS.C6.NW68	Proline substitution	Corbett	2/29/16	V804P

Stabilizing mutations were added to MERS England strain spike protein (MERS.C6.NW0) to stabilize the protein in prefusion form. Preliminary studies reveal mutations V1060P and L1061P (yellow) increase the stability of MERS spike trimer, but additional stabilizing mutations may be needed for complete stabilization. Additional mutations, contributed by the Graham lab (blue), McLellan lab (black) and Ward (lab), will be tested in the future. Corresponding sequences are attached in excel format.

1. Kirchdoerfer, R.N., et al., *Pre-fusion structure of a human coronavirus spike protein.* Nature, 2016. **531**(7592): p. 118-121.
2. Qian, Z., et al., *Identification of the Receptor-Binding Domain of the Spike Glycoprotein of Human Betacoronavirus HKU1.* J Virol, 2015. **89**(17): p. 8816-27.
3. McLellan, J.S., et al., *Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus.* Science, 2013. **342**(6158): p. 592-8.
4. Sanders, R.W., et al., *A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies.* PLoS Pathog, 2013. **9**(9): p. e1003618.
5. Krarup, A., et al., *A highly stable prefusion RSV F vaccine derived from structural analysis of the fusion mechanism.* Nat Commun, 2015. **6**: p. 8143.
6. Woo, P.C., et al., *Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia.* J Virol, 2005. **79**(2): p. 884-95.
7. de Wit, E., et al., *SARS and MERS: recent insights into emerging coronaviruses.* Nat Rev Microbiol, 2016.
8. Al-Tawfiq, J.A., A.S. Omrani, and Z.A. Memish, *Middle East respiratory syndrome coronavirus: current situation and travel-associated concerns.* Front Med, 2016. **10**(2): p. 111-9.
9. Menachery, V.D., et al., *SARS-like WIV1-CoV poised for human emergence.* Proc Natl Acad Sci U S A, 2016. **113**(11): p. 3048-53.
10. Wang, L., et al., *Evaluation of candidate vaccine approaches for MERS-CoV.* Nat Commun, 2015. **6**: p. 7712.

Manuscript

Prefusion structure of a human coronavirus spike protein

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HKU1 is a human betacoronavirus that causes mild yet prevalent respiratory disease[1] and is related to the zoonotic SARS[2] and MERS[3] betacoronaviruses that have high fatality rates and pandemic potential. Cell tropism and host range is determined in part by the coronavirus spike (S) protein[4]. As the largest known class I membrane fusion protein, its size and extensive glycosylation have hindered structural studies of the full ectodomain, thus preventing a molecular understanding of its function and limiting development of effective interventions. Here we present the 4.0 Å resolution structure of the HKU1 S protein trimer determined using single-particle cryo-electron microscopy. In the prefusion conformation, the receptor-binding subunits, S1, rest atop the fusion-mediating subunits, S2, preventing their conformational rearrangement. Surprisingly, the S1 C-terminal domains are interdigitated and form extensive quaternary interactions that occlude surfaces known to bind protein receptors in other coronaviruses. The C-terminal domains are connected to two previously unidentified sub-domains, one of which contains the N-terminus of S2. This work provides a structural basis to support a model of membrane fusion mediated by progressive S protein destabilization through receptor binding and proteolytic cleavage. In addition to these mechanistic insights, these studies should serve as a foundation for the structure-based design of betacoronavirus vaccine immunogens and isolation of therapeutic antibodies from convalescent patients.

Human coronavirus HKU1 (HCoV-HKU1)[1] is an airborne betacoronavirus that circulates globally and causes respiratory illness that can be severe in children, the elderly and adults with underlying health conditions[5]. Two other betacoronaviruses are associated with even more severe disease: severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 with a 10% mortality rate[6] and Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012 with

a 36% mortality rate[7]. Both viruses emerged from animal reservoirs due in part to the ability of the viral spike (S) glycoprotein to recognize human receptors[4].

Coronavirus S proteins are processed into S1 and S2 subunits by host proteases[8]. Like other class I viral fusion proteins, the two subunits trimerize and fold into a metastable prefusion conformation. Similarly, the S1 subunit is responsible for receptor binding while the S2 subunit mediates membrane fusion. Coronaviruses typically possess two domains within S1 capable of binding to host receptors: an N-terminal domain (NTD) and a C-terminal domain (CTD), with the latter recognizing protein receptors for SARS-CoV and MERS-CoV[9,10]. Although these individual domains have been structurally characterized, they represent only a portion of the S protein, and their organization within the functionally active spike has not yet been determined, preventing a mechanistic understanding of S protein function.

Here, we present the structure of the HKU1 S protein ectodomain determined using cryo-electron microscopy (cryo-EM) to 4.0 Å resolution (Fig. 1a and Extended Data Fig. 1 and 2 and Table 1). The reconstructed S trimer resembles an inverted bell and is ~150 Å tall with a maximum radius of ~70 Å near the membrane-distal apex. The S1 subunit adopts an extended conformation with short linkers between domains and sub-domains (Fig. 1b). The S1 NTD (amino acids 14–297) has strong structural and sequence homology to the Bovine coronavirus (BCoV) S1 NTD (Extended Data Fig. 3), which recognizes acetylated sialic acid residues on cell-surface proteins[11]. The glycan-binding site in the BCoV S1 NTD is conserved in the HKU1 S1 NTD and is located at the apex of the trimer directed toward target cells, suggesting that it too functions as a glycan-binding domain. Indeed, HKU1 S1 was recently shown to bind *O*-acetylated sialic acids on host cells, and these sugars were required for efficient infection of primary human airway epithelial cultures[12].

45 The HKU1 S1 CTD (amino acids 325–605) consists of a structurally conserved core connected
46 to a large, variable loop (HKU1 S amino acids 428–587)[13] (Extended Data Fig 4). For SARS-CoV and
47 MERS-CoV, this loop interacts with the host protein receptors ACE2 and DPP4, respectively[14,15]. A
48 protein receptor has not yet been identified for HKU1, but the S1 CTD was recently identified as the
49 receptor-binding site[16]. The HKU1 S1 CTD is located at the trimer apex close to the three-fold axis. In
50 the HKU1 S cryo-EM map, the core of the S1 CTD is well resolved, including the regions structurally
51 homologous to those of the SARS-CoV and MERS-CoV S1 CTDs[14,15]. However, some regions of the
52 variable loops distal to the core appear disordered (Extended Data Fig. 4). The structurally conserved
53 CTD core interacts near the three-fold trimer axis with the other two S1 CTD cores, and with one NTD
54 from an adjacent protomer. The domain swapping between protomers results in the appearance of the
55 trimer being woven together when viewed looking down toward the viral membrane (Fig. 2a).
56 Structural alignment of the SARS-CoV and MERS-CoV CTD–receptor complexes[14,15] with the HKU1
57 prefusion S protein reveals that the protein-receptor-binding surface of the S1 CTD is buried in the
58 HKU1 S protein trimer and is therefore incapable of making equivalent interactions without some
59 initial breathing and transient exposure of these domains (Fig. 2b). These findings are consistent with a
60 model where conformational changes upon receptor binding destabilize the trimeric S protein to
61 promote the membrane-fusion process.
62 The HKU1 S1 also contains two smaller, largely β-sheet sub-domains (which we term SD-1
63 and SD-2) that lack significant homology to previously determined structures (Fig. 2c and d). These
64 sub-domains are primarily composed of S1 amino acid sequences following the CTD. However,
65 stretches of amino acids preceding the CTD as well as S2 residues adjacent to the S1/S2 cleavage site
66 also contribute to the sub-domains. This complex folding of elements dispersed throughout the primary
67 sequence may allow receptor-induced conformational changes in the CTD to be transmitted to other

parts of the structure. Indeed, the parallel β-strands in these sub-domains may dissociate during the conformational changes that occur during membrane fusion, analogous to other class I viral fusion proteins[17].

In contrast to other viral fusion proteins such as influenza hemagglutinin (HA)[18] or HIV envelope (Env)[19,20], the HKU1 S1 subunits are rotated about the trimeric three-fold axis with respect to the S2 subunits, causing the S1 subunit from one protomer to sit atop the S2 subunit of an adjacent protomer (Extended Data Fig. 5). The NTD of S1 makes no direct contacts with S2. However, the S1 CTD and the two S1 sub-domains make extensive contacts with the membrane-distal portion of the S2 subunit. Similar to influenza HA and HIV Env, a region in the HKU1 S1 CTD (amino acids 371–380) caps the S2 central helix, thereby preventing the fusion machinery from springing into action (Extended Data Fig. 6).

Proteolytic cleavage of coronavirus S proteins by host proteases plays a critical role in the entry process[8]. Though mutated in the protein construct used here and disordered in the density map, the HKU1 S furin-cleavage site at the S1/S2 junction lies in a loop of SD-2 (Fig. 3). Examination of cleaved HKU1 S protein using negative-stain electron microscopy did not reveal any large conformational differences (Extended Data Fig. 7), suggesting that cleavage of the S1/S2 site is not required to adopt the prefusion conformation. Furin cleavage would leave a single S2 β-strand participating in the SD-2 β-sheets (Fig. 2d). Notably, the furin-recognition sequence is not conserved across all coronaviruses[8] and several coronavirus S proteins are reported to be cleaved only after receptor recognition, as is the case for SARS-CoV[21]. SARS-CoV S is targeted by trypsin-like proteases at Arg677[22], a site equivalent to the HKU1 S1/S2 furin-cleavage site. SARS-CoV S is also reported to be cleaved by cathepsins at amino acid 678[23] (HKU1 S homologous amino acid 772). By

homology to the HKU1 S protein structure presented here, this cathepsin cleavage would occur after SD-2, closer to the viral membrane (Fig. 3).

Many class I viral fusion proteins possess fusion peptides adjacent to their cleavage sites. In contrast, coronavirus S proteins are unusual in having a fusion peptide located more than 100 amino acids C-terminal to the S1/S2 cleavage site[24]. However, coronaviruses also have a secondary cleavage site, termed S2' (Arg900)[8], adjacent to the viral fusion peptide (amino acids 901–918) (Fig. 3b). Cleavage at S2' by furin- or trypsin-like proteases likely follows S1/S2 cleavage and may not occur until host-receptor engagement and viral endocytosis[8]. Based on the HKU1 S protein structure, cleavage at the coronavirus S2' position would not likely cause the resulting protein fragments to dissociate immediately from the prefusion trimeric S protein. The protein region between the S1/S2 and S2' cleavage sites (HKU1 S amino acids 758–900) is partially buried beneath the heptad repeat 1 (HR1) helices suggesting that this peptide is not released from the trimeric S protein prior to conformational rearrangement of the S2 fusion machinery.

As in all class I viral fusion proteins, the coronavirus S2 subunit contains four elements required for membrane fusion: a fusion peptide or loop, HR1, HR2, and a viral transmembrane domain[17,18,25]. Refolding of HR1 into a long α-helix thrusts the fusion peptide into the host-cell membrane, and as the two heptad repeats interact with each other to form a coiled-coil. This process results in S2 adopting a hairpin conformation and the host and viral membranes are brought into close proximity. The HKU1 S fusion peptide, conserved among coronavirus S proteins[24], begins at amino acid 901 and is located on the exterior of the trimer and adjacent to the putative S2' cleavage site, which remains uncleaved in the sample used for structure determination here. In the HKU1 S structure, density for the fusion peptide is visible starting at amino acid 902. The fusion peptide forms a short helix and a loop where most of the hydrophobic amino acids are buried in an interface with S2.

113 Although this HKU1 spike is uncleaved at both the S1/S2 boundary and S2', it is possible that protease
114 cleavage at the S2' site may alter the conformation of the fusion peptide on the surface of S2 similar to
115 influenza HA[18]. However, unlike influenza HA where the C-terminus of the fusion peptide is only 14
116 amino acids away from the N-terminus of its HR1, the fusion peptide of HKU1 S is 60 amino acids
117 away from its HR1. This span of protein contains four short α-helices and several longer regions
118 lacking regular secondary structure. This intervening sequence is also buried beneath SD-2 and the S2'
119 cleavage site, suggesting that protease cleavage may affect the proclivity of S for undergoing the
120 transition to the postfusion conformation.

121 In coronaviruses, the heptad repeats are unusually large with HR1 encompassing more than 90
122 amino acids[25]. In the cryo-EM structure, HR2 is located at the base of the HKU1 S protein near the
123 viral membrane, but is poorly ordered, precluding unambiguous assignment of the residues. However,
124 HR1 is well ordered and arranged along the length of the S2 subunit forming four short helices and
125 part of the central three-helix bundle. The coronavirus arrangement of HR1 is similar to that of
126 influenza HA, where HR1 is organized as two helices connected by a long loop[18]. In the influenza HA
127 postfusion conformation, HR1 undergoes a loop-to-helix transition to form a single long α-helix[17].
128 The postfusion six-helix bundle structures of SARS-CoV and MERS-CoV S2 heptad repeats[26,27] reveal
129 that coronaviruses also undergo a similar transition (Fig 3c). However, comparison of the coronavirus
130 pre- and postfusion HR1 regions shows that during this conformational change the coronavirus spike
131 must carry out five such loop-to-helix transitions, highlighting the complexity of S proteins relative to
132 other class I fusion proteins. In addition, the membrane distal regions of the prefusion S2 central three-
133 helix bundle (S2 amino acids 1070–1076), which is the C-terminal portion of HR1, are splayed
134 outwards from the three-fold axis (Extended Data Fig. 8). In the available coronavirus postfusion
135 HR1–HR2 structures, this portion of HR1 forms a tight three-helix bundle[26,27]. This observation

136 indicates that the α-helices of the prefusion central core must come together during fusion. Formation
137 of the three-helix bundle may be prevented by interactions between the C-terminal end of the S2 HR1
138 and the S1 CTD in the prefusion conformation (Extended Data Fig 6 and 8). Disruption of this
139 interaction through receptor-induced conformational changes in S1 provides an additional means by
140 which receptor binding can initiate S2-mediated membrane fusion.

141 The HCoV-HKU1 S trimer in a prefusion conformation is the largest class I viral fusion protein
142 structure determined to date (Fig. 4 and Extended Data Fig. 9). Since betacoronavirus S proteins are
143 similar in size and have a conserved domain organization, our findings should be generally applicable
144 to other betacoronaviruses, including SARS-CoV and MERS-CoV. Our studies provide a structural
145 basis for S protein function where the prefusion S protein is progressively matured and destabilized by
146 binding of a protein receptor and cleavage by host proteases. These changes would free the viral fusion
147 peptide and allow the HR1 region of the prefusion spike to straighten and transition to a long α-helix
148 extending towards the host membrane. The conformational changes in HR1 would release the fusion
149 peptide from the side of the S2 subunit, allowing insertion into host membranes. The structure and
150 mechanistic insights presented here should enable engineering of stabilized coronavirus S proteins for
151 testing conformationally relevant, prefusion S vaccine immunogens against current and emerging
152 betacoronaviruses, similar to recent efforts for other viral fusion proteins[28,29]. This work also acts as a
153 springboard for future studies to define mechanisms of antibody recognition and neutralization, which
154 will lead to an improved understanding of coronavirus immunity.

References:

1. Woo, P. C. *et al.* Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* (2005) **79**, 884-895.

2. Christian, M. D., Poutanen, S. M., Loutfy, M. R., Muller, M. P. & Low, D. E. Severe acute respiratory syndrome. *Clin Infect Dis* (2004) **38**, 1420-1427.

3. Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. & Fouchier, R. A. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* (2012) **367**, 1814-1820.

4. Graham, R. L. & Baric, R. S. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. *J Virol* (2010) **84**, 3134-3146.

5. van der Hoek, L. Human coronaviruses: what do they cause? *Antivir Ther* (2007) **12**, 651-658.

6. Cherry, J. D. The chronology of the 2002-2003 SARS mini pandemic. *Paediatr Respir Rev* (2004) **5**, 262-269.

7. Zumla, A., Hui, D. S. & Perlman, S. Middle East respiratory syndrome. *Lancet* (2015) **386**, 995-1007.

8. Millet, J. K. & Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res* (2015) **202**, 120-134.

9. Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* (2003) **426**, 450-454.

10. Mou, H. *et al.* The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. *J Virol* (2013) **87**, 9379-9383.

11. Peng, G. *et al.* Crystal structure of bovine coronavirus spike protein lectin domain. *J Biol Chem* (2012) **287**, 41931-41938.

12. Huang, X. *et al.* Human Coronavirus HKU1 Spike Protein Uses O-Acetylated Sialic Acid as an Attachment Receptor Determinant and Employs Hemagglutinin-Esterase Protein as a Receptor-Destroying Enzyme. *J Virol* (2015) **89**, 7202-7213.

13. Li, F. Receptor recognition mechanisms of coronaviruses: a decade of structural studies. *J Virol* (2015) **89**, 1954-1964.

14. Li, F., Li, W., Farzan, M. & Harrison, S. C. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* (2005) **309**, 1864-1868.

15. Lu, G. *et al.* Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. *Nature* (2013) **500**, 227-231.

16. Qian, Z. *et al.* Identification of the Receptor-Binding Domain of the Spike Glycoprotein of Human Betacoronavirus HKU1. *J Virol* (2015) **89**, 8816-8827.

17. Bullough, P. A., Hughson, F. M., Skehel, J. J. & Wiley, D. C. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* (1994) **371**, 37-43.

18. Wilson, I. A., Skehel, J. J. & Wiley, D. C. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature* (1981) **289**, 366-373.

19. Julien, J. P. *et al.* Crystal structure of a soluble cleaved HIV-1 envelope trimer. *Science* (2013) **342**, 1477-1483.

20. Lyumkis, D. *et al.* Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. *Science* (2013) **342**, 1484-1490.

21. Matsuyama, S. *et al.* Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol* (2010) **84**, 12658-12664.

22. Li, F. *et al.* Conformational states of the severe acute respiratory syndrome coronavirus spike protein ectodomain. *J Virol* (2006) **80**, 6794-6800.

23. Bosch, B. J., Bartelink, W. & Rottier, P. J. Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide. *J Virol* (2008) **82**, 8887-8890.

24. Madu, I. G., Roth, S. L., Belouzard, S. & Whittaker, G. R. Characterization of a highly conserved domain within the severe acute respiratory syndrome coronavirus spike protein S2 domain with characteristics of a viral fusion peptide. *J Virol* (2009) **83**, 7411-7421.

25. Bosch, B. J., van der Zee, R., de Haan, C. A. & Rottier, P. J. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* (2003) **77**, 8801-8811.

26. Duquerroy, S., Vigouroux, A., Rottier, P. J., Rey, F. A. & Bosch, B. J. Central ions and lateral asparagine/glutamine zippers stabilize the post-fusion hairpin conformation of the SARS coronavirus spike glycoprotein. *Virology* (2005) **335**, 276-285.

27. Lu, L. *et al.* Structure-based discovery of Middle East respiratory syndrome coronavirus fusion inhibitor. *Nat Commun* (2014) **5**, 3067.

28. McLellan, J. S. *et al.* Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. *Science* (2013) **342**, 592-598.

29. Sanders, R. W. *et al.* A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog* (2013) **9**, e1003618.

30. Lee, P. S. *et al.* Receptor mimicry by antibody F045-092 facilitates universal binding to the H3 subtype of influenza virus. *Nat Commun* (2014) **5**, 3614.

31. Suloway, C. *et al.* Automated molecular microscopy: the new Leginon system. *J Struct Biol* (2005) **151**, 41-60.

32. Lander, G. C. *et al.* Appion: an integrated, database-driven pipeline to facilitate EM image processing. *J Struct Biol* (2009) **166**, 95-102.

33. Voss, N. R., Yoshioka, C. K., Radermacher, M., Potter, C. S. & Carragher, B. DoG Picker and TiltPicker: software tools to facilitate particle selection in single particle electron microscopy. *J Struct Biol* (2009) **166**, 205-213.

34. Sorzano, C. O. *et al.* XMIPP: a new generation of an open-source image processing package for electron microscopy. *J Struct Biol* (2004) **148**, 194-204.

35. Yang, Z., Fang, J., Chittuluru, J., Asturias, F. J. & Penczek, P. A. Iterative stable alignment and clustering of 2D transmission electron microscope images. *Structure* (2012) **20**, 237-247.

36. Ludtke, S. J., Baldwin, P. R. & Chiu, W. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J Struct Biol* (1999) **128**, 82-97.

37. Li, X. *et al.* Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat Methods* (2013) **10**, 584-590.

38. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J Struct Biol* (2003) **142**, 334-347.

39. Ogura, T., Iwasaki, K. & Sato, C. Topology representing network enables highly accurate classification of protein images taken by cryo electron-microscope without masking. *J Struct Biol* (2003) **143**, 185-200.

40. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J Struct Biol* (2012) **180**, 519-530.

41. Scheres, S. H. & Chen, S. Prevention of overfitting in cryo-EM structure determination. *Nat Methods* (2012) **9**, 853-854.

42. Webb, B. & Sali, A. Comparative Protein Structure Modeling Using MODELLER. *Curr Protoc Bioinformatics* (2014) **47**, 5.6.1-5.6.32.

43. Pettersen, E. F. *et al.* UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* (2004) **25**, 1605-1612.

44. DiMaio, F. *et al.* Atomic-accuracy models from 4.5-A cryo-electron microscopy data with density-guided iterative local refinement. *Nat Methods* (2015) **12**, 361-365.

45. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* (2010) **66**, 486-501.

46. Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* (2010) **66**, 213-221.

47. Schrodinger, L. The PyMOL Molecular Graphics System, Version 1.5.0.4. (2012).

48. Barad, B. A. *et al.* EMRinger: side chain-directed model and map validation for 3D cryo-electron microscopy. (2015) **12**, 943-946.

49. Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* (2010) **66**, 12-21.

Supplementary Information is available in the online version of the paper.

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Author Contributions H.M.Y. and K.S.C. designed the HKU1 S expression constructs. N.W. expressed and purified the proteins. C.A.C., H.L.T., and J.P. prepared samples for cryoEM, collected

images, and processed the data. R.N.K. and C.A.C built and refined the model. B.S.G., J.S.M., and A.B.W. conceived of the project. R.N.K, C.A.C., N.W., B.S.G, J.S.M and A.B.W analyzed the results and wrote the manuscript, with all authors editing and approving the final manuscript.

Author Information The cryo-EM map of the HKU1 spike ectodomain has been deposited in the EMDB under accession number XXXX and the coordinates of the HKU1 spike ectodomain structure have been deposited in the PDB under accession number YYYY. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.S.M. (Jason.S.McLellan@Dartmouth.edu) and A.B.W. (ABWard@Scripps.edu).

Figures and Tables:

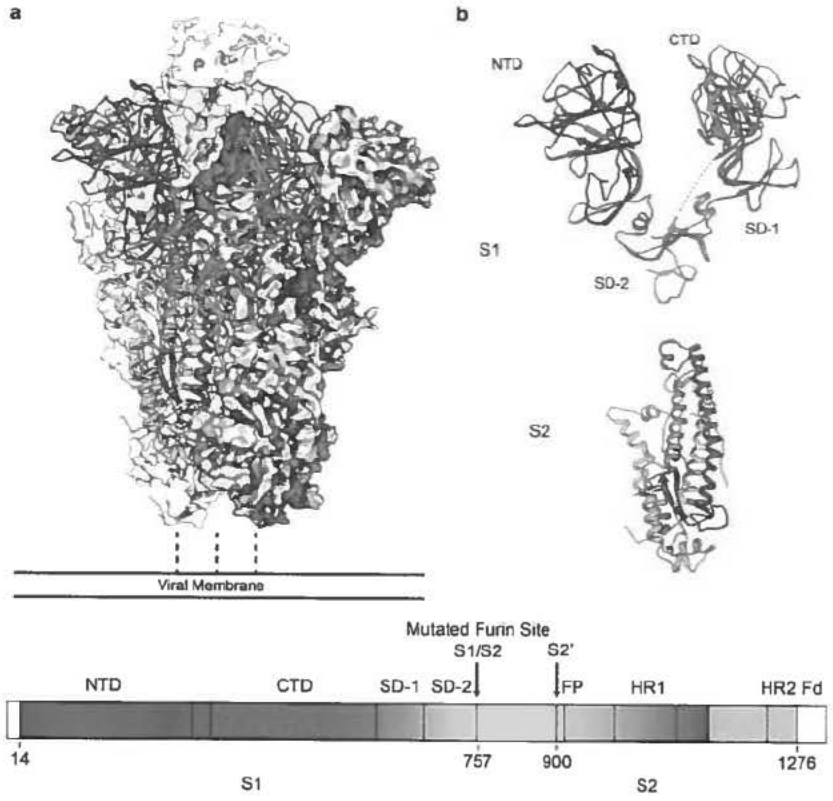


Figure 1 | Structure of the HKU1 prefusion spike ectodomain. a, A single protomer of the trimeric S protein is shown in cartoon representation colored as a rainbow from the N- to C-terminus (blue to red) with the reconstructed EM density of remaining protomers shown in white and grey. **b**, The S1 subunit is composed of the NTD and CTD as well as two sub-domains (SD-1 and SD-2). The S2 subunit contains the coronavirus fusion machinery and is primarily α-helical. *inset*, Domain architecture of the HKU1 S protein colored as in (**a**).

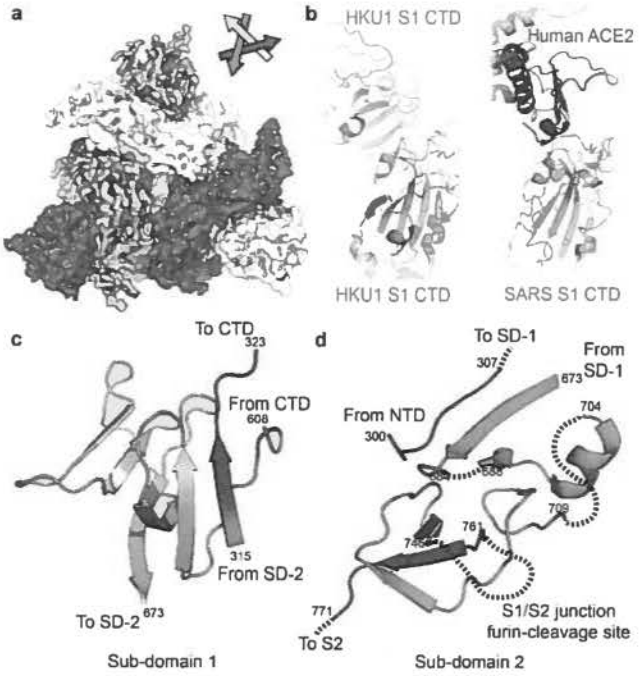


Figure 2 | Architecture of the HKU1 S1 subunit. a, The NTD and CTD of HKU1 S are interdigitated at the trimer apex. EM density corresponding to each S1 protomer is shown as a surface. **b,** The HKU1 S1 CTD forms quaternary interactions with an adjacent CTD using a surface similar to that used by SARS CTD to bind its receptor, ACE2[14]. **c,** Sub-domain 1 is a collection of β-sheets contributed by amino acid residues before and after the S1 CTD. **d,** Sub-domain 2 is primarily composed of S1 sequence C-terminal to the CTD, but it also contains a short peptide following the NTD as well as the N-terminal strand of S2, which follows the S1/S2 furin-cleavage site.

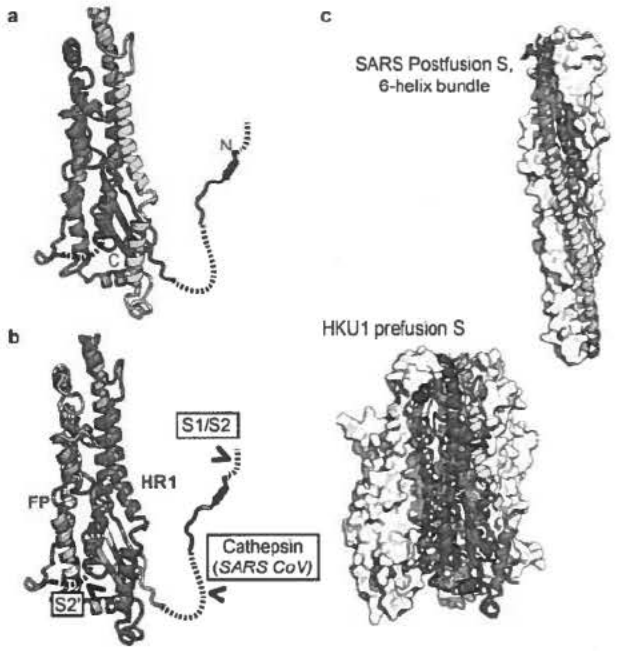


Figure 3 | HKU1 S2 subunit fusion machinery. a, The HKU1 S2 subunit is colored as a rainbow from the N-terminal β-strand (blue), which participates in S1 sub-domain 2, to a C-terminal β-strand (red) prior to HR2. **b**, The HKU1 S2 structure contains the fusion peptide (FP) and a long heptad repeat (HR1). Protease-recognition sites important for maturation and function of the S protein are indicated within disordered regions of the protein (dashed lines). **c**, A comparison of HR1 in the HKU1 prefusion conformation and the SARS postfusion six-helix bundle[26]. Five HR1 α-helices are colored as a rainbow from blue to red, N- to C-terminus, respectively. The structures are oriented so as to position similar portions of the central helix (colored red).

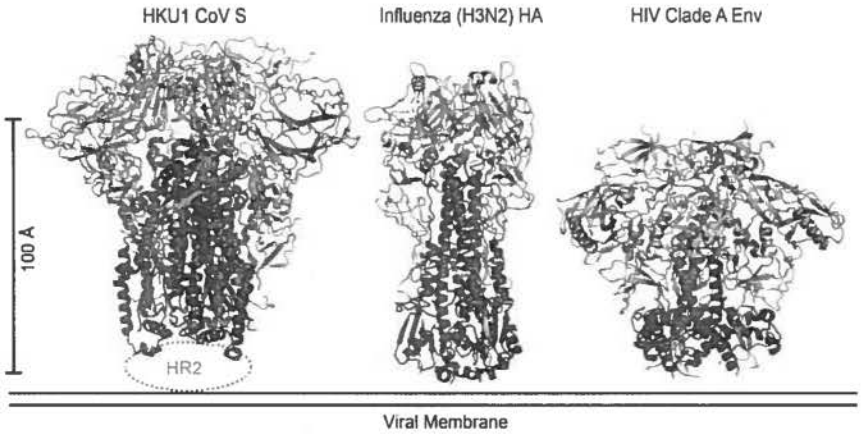


Figure 4 | Comparison of structurally related class I viral fusion proteins. The viral fusion proteins from coronaviruses, influenza and HIV-1 are cleaved into receptor-binding subunits (pink, light green, light blue) and the viral fusion machinery (dark red, dark green, blue)[18-20,30].

METHODS

Protein expression and purification. A mammalian-codon-optimized gene encoding HKU1 S (isolate N5, NCBI accession Q0ZME7) residues 1–1276 with a C-terminal T4 fibritin trimerization domain, a HRV3C cleavage site, and a 6x His-tag was synthesized and subcloned into the eukaryotic expression vector pVRC8400. The S1/S2 furin-recognition site RRKRR was mutated to GGSGS to generate the uncleaved construct used for cryoEM studies. Three hours after this plasmid was transfected into FreeStyle 293-F cells (Invitrogen), kifunensine was added to a final concentration of 5 μM. Cultures were harvested after six days, and protein was purified from the media using Ni-NTA Superflow resin (Qiagen). The buffer was then exchanged using a HiPrep 26/10 desalting column (GE Healthcare Biosciences) from a high-imidazole elution buffer to a low pH buffer (20 mM Bis-Tris pH 6.5, 150 mM NaCl). Afterward, Endoglycosidase H (10% w/w) and HRV3C protease (1% w/w) were added to the protein and the reaction was incubated overnight at 4°C. The digested protein was further purified using a Superose 6 16/70 column (GE Healthcare Biosciences).

The furin-cleaved HKU1 S construct analyzed by negative-stain EM was similar to the one described above except that it encoded residues 1–1249 and contained the wild-type RRKRR furin-recognition site. Expression and purification were also similar, except that a plasmid expressing furin was co-transfected into the FreeStyle 293-F cells to ensure complete processing of the protein.

Sample preparation for negative-stain electron microscopy. HKU1 samples were placed directly onto 400 copper mesh grids and then stained with 1% uranyl formate. Tris-buffered saline (TBS) was used as buffer if dilution was necessary.

Negative-stain electron microscopy data collection. Grids were loaded into a Tecnai T12 Spirit operating at 120 keV and imaged using a Tietz TemCam-F416 CMOS at 52,000 × magnification at a nominal defocus of ~1.5 μM. Micrographs were collected using Leginon[31] and processed within

Appion[32]. Particles were picked using a difference-of-Gaussians approach[33]. Stacks were made with picked particles and 2D classification was undertaken in XMIPP[34]. Classes that did not represent views of HKU1 were discarded giving a final stack of 5,281 particles. Final 2D stacks were made using ISAC[35] and a 3D model was generated using EMAN[36].

Sample preparation for cryo-electron microscopy. Sample solution (3 μL) was applied to the carbon face of a CF-2/2-4C C-Flat grid (Electron Microscopy Sciences, Protochips, Inc.) that had been plasma cleaned for five seconds using a mixture of Ar/O_2 (Gatan Solarus 950 Plasma system). The grid was then manually blotted and immediately plunged into liquid ethane using a manual freeze plunger.

Cryo-electron microscopy data collection. Micrographs were collected via the Leginon interface on a FEI Titan Krios operating at 300 kV mounted with a Gatan K2 direct electron detector[31]. Each micrograph was collected in counting mode at 22,500 × nominal magnification resulting in a calibrated pixel size of 1.31 Å/pix at the object level. A dose rate of ~10 e^-/((cam pix)×s) was used; exposure time was 200 ms per frame. The data collection resulted in a total of 1,049 micrographs. Total dose for these micrographs was 57 $e^-/Å^2$. The nominal defocus range used was -1.0 to -3.5 μm.

Cryo-Electron Microscopy Data processing. All of the collected frames were aligned[37], CTF estimation was carried out using CTFFIND3 [38], and particles were picked employing a difference-of-Gaussians approach[33]. Reference-free 2D classification was performed employing iterative multivariate statistical analysis/multi-reference alignment using a binning factor of 2 to remove amorphous particles[39]. After 2D classification, unbinned selected particles were refined in RELION version 1.4b1[40,41] against the HKU1 negative-stain density map filtered to 60 Å resolution imposing C3 symmetry. This refinement was followed by particle polishing and refinement of the resulting realigned, B-factor-weighted and signal-integrated particles. The resolution of the final map was 4.04

Å at an FSC cutoff of 0.143. The FSC was calculated using a soft-edged mask with a Gaussian falloff, encompassing the entire structure.

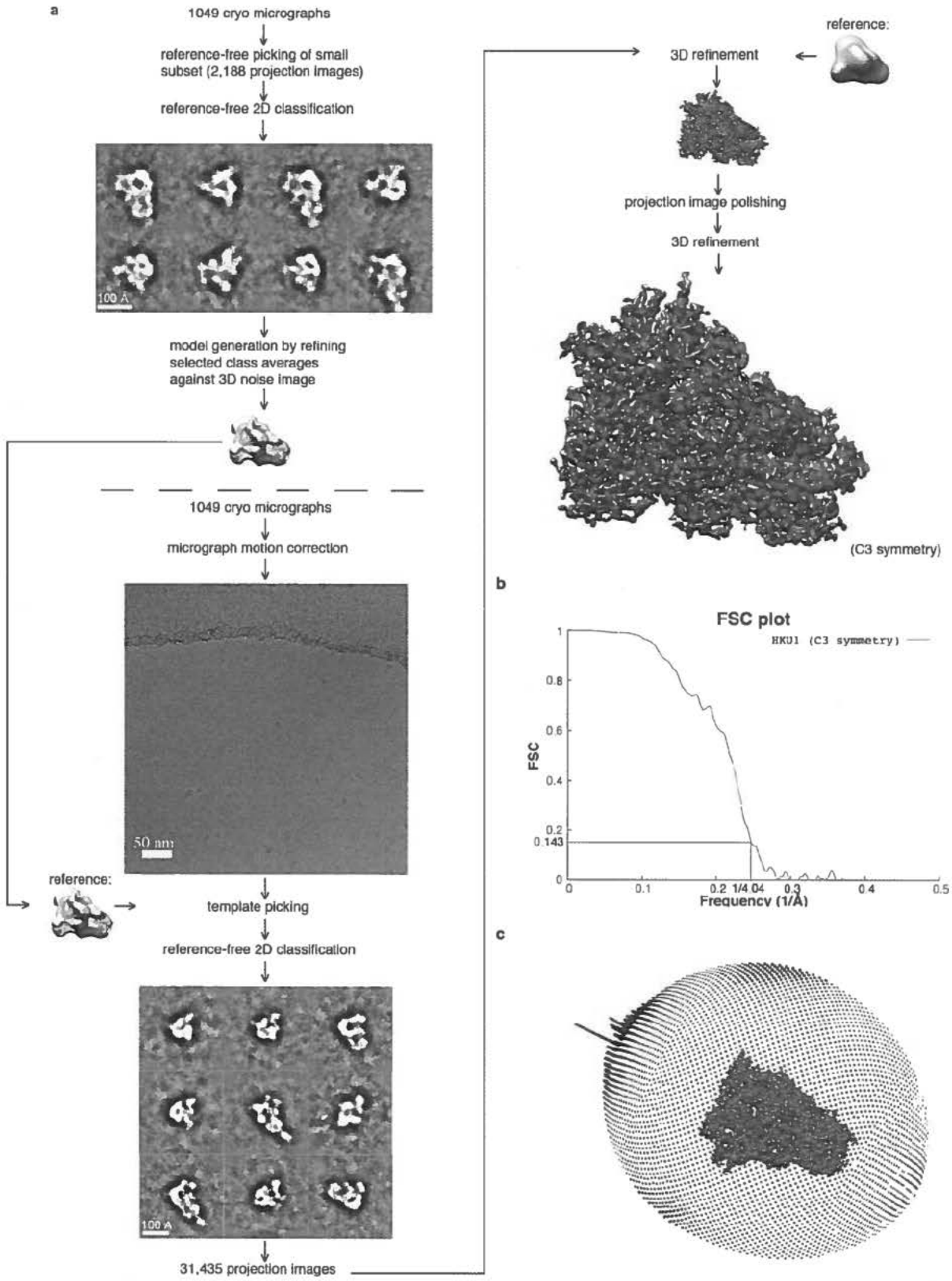
Model Building and Refinement

Initial models of the S1 NTD were generated using the Modeller[42] homology modeling tool in UCSF Chimera[43] with the BoCoV NTD (PDB 4H14)[11] as a template. The NTD homology model was docked into the HKU1 EM density and refined with Rosetta density-guided iterative local refinement[44] while imposing C3 symmetry. Rosetta output models were clustered based on pairwise RMSD using a cluster radius of 2.15 Å. The lowest energy model from the largest cluster was selected for additional refinement. This model and the conserved core CTD from SARS CoV (PDB 2AJF)[14] were used as starting structures for model building and refinement. These starting models and the remaining HKU1 protein sequence was modeled manually using COOT[45] and refined using Phenix real space refinement[46]. Figures were produced in the PyMol[47] or UCSF Chimera[43] software packages.

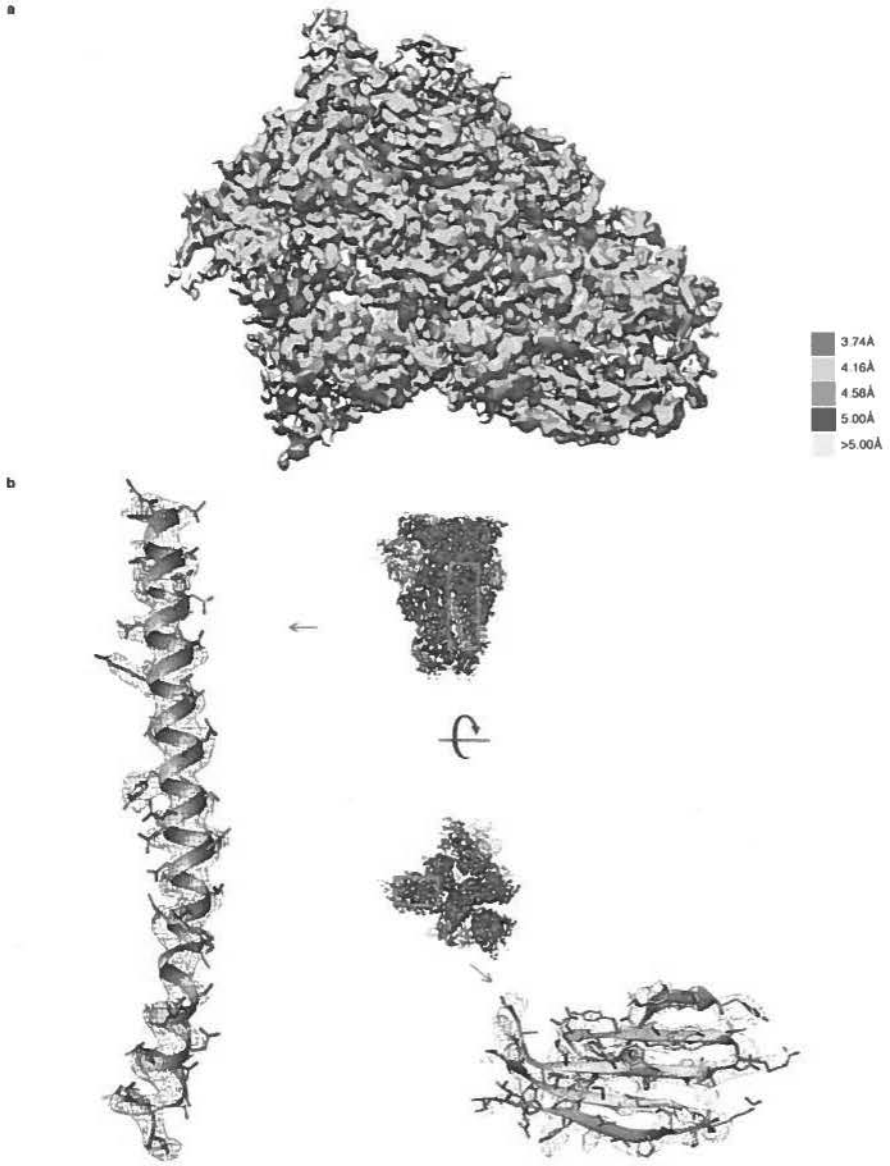
Extended Data Tables and Figures

Measure	Value
Resolution	4.0
Chimera CC[43]	0.88
Clash score	20.6
EM Ringer Score[48]	3.0
Molprobity[49]	2.5
Ramachandran (%)	
Favored	80.0
Outliers	0.2

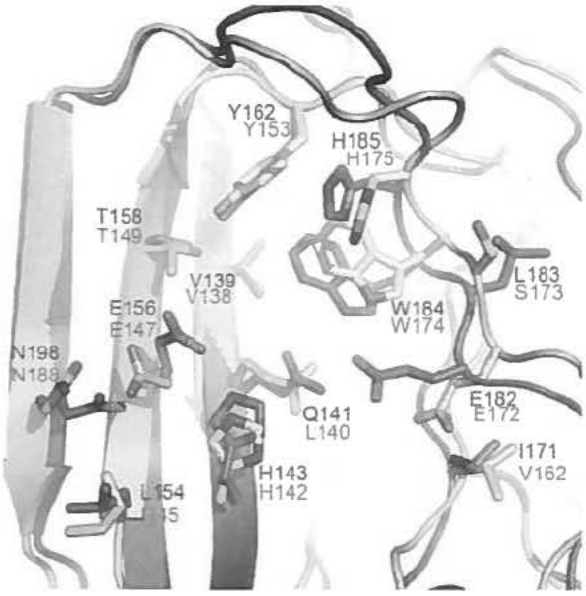
Table 1 | Structure statistics.


a
1049 cryo micrographs
reference-free picking of small subset (2,188 projection images)
reference-free 2D classification
100 Å
model generation by refining selected class averages against 3D noise image
1049 cryo micrographs
micrograph motion correction
50 nm
reference:
template picking
reference-free 2D classification
100 Å
31,435 projection images
reference:
3D refinement
projection image polishing
3D refinement
(C3 symmetry)
b
FSC plot
HKU1 (C3 symmetry)
FSC
1
0.8
0.6
0.4
0.2
0.143
0
0
0.1
0.2 1/4.04
0.3
0.4
0.5
Frequency (1/Å)
c

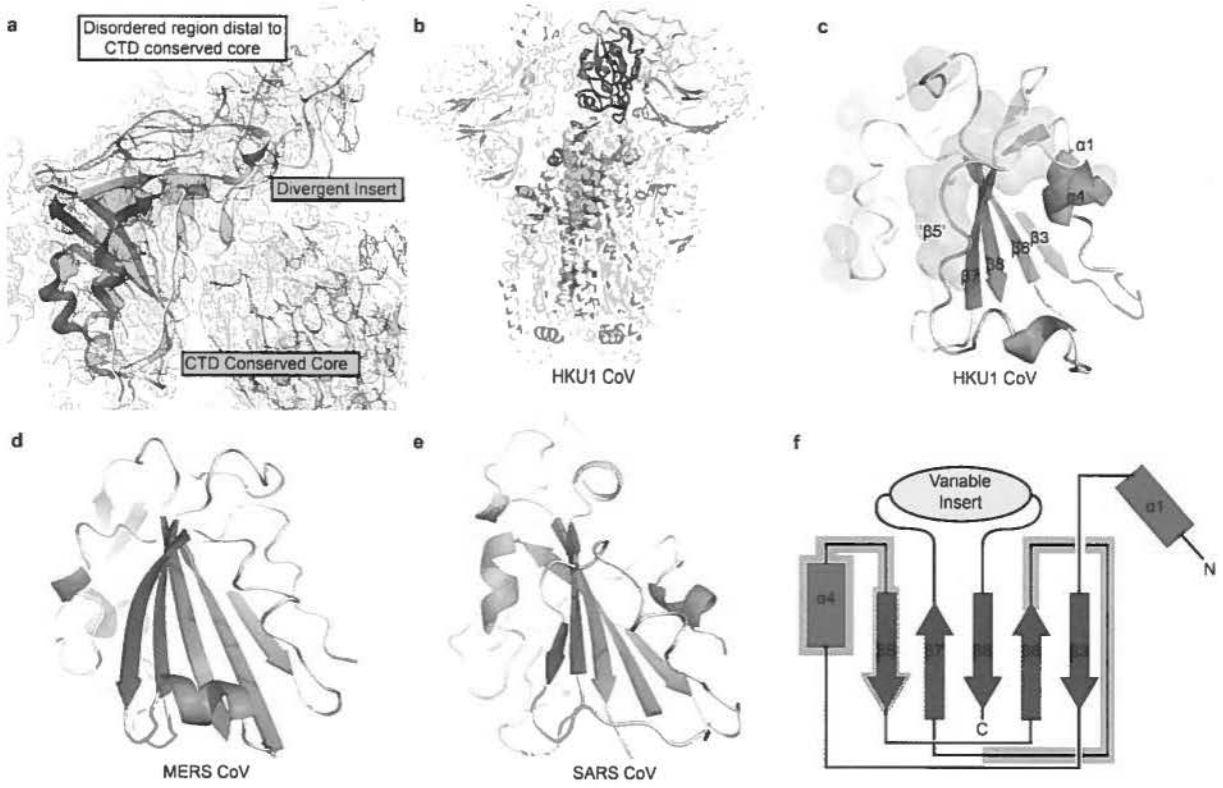
Extended Data Figure 1 | Data processing flowchart. a, Processing resulting in density map of prefusion HKU1 spike glycoprotein at 4.04 Å resolution. **b**, FSC plot illustrating correlation between two volumes refined independently from two distinct half sets of raw data. A final resolution of 4.04 Å is indicated in the plot. **c**, Angular distribution of raw data within the data set. A slight, but within normal range, over-representation of top and side views was observed (tall red bars).



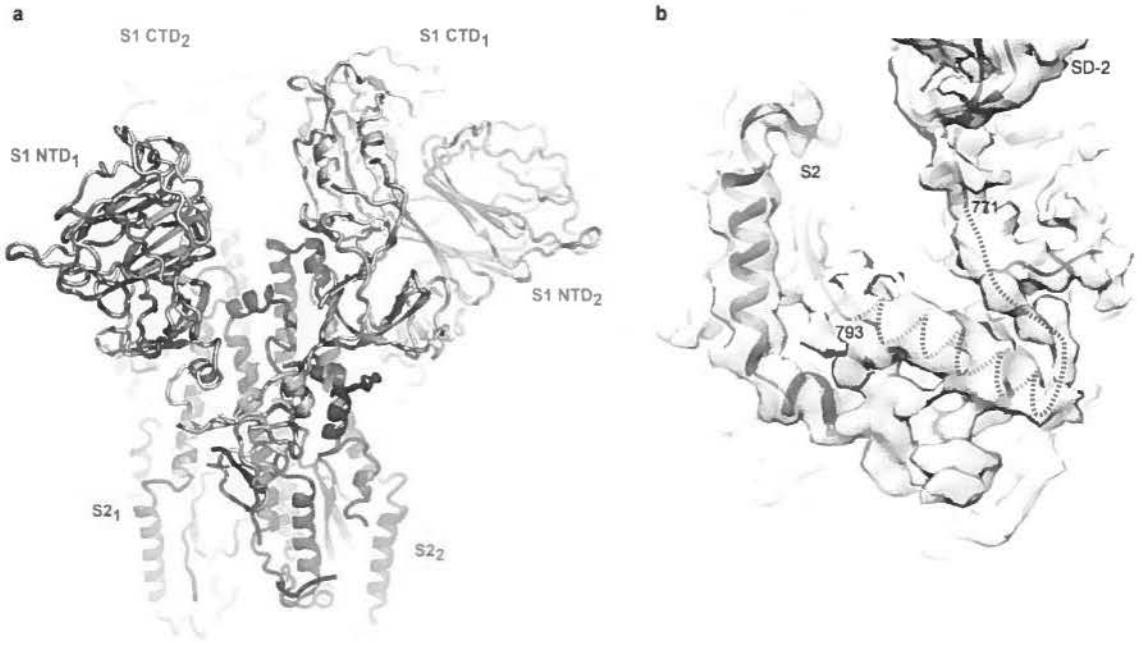
Extended Data Figure 2 | Resolution of the prefusion HKU1 S density map. a, Local resolution within the EM density map. Resolution ranges from 3.74 Å in stable internal secondary structures to greater than 5.00 Å in flexible peripheral loops. **b**, Close-ups of secondary-structure densities. To the left is displayed the central α-helix of an S2 monomer and to the right is a β-sheet from the NTD domain in an S1 monomer.



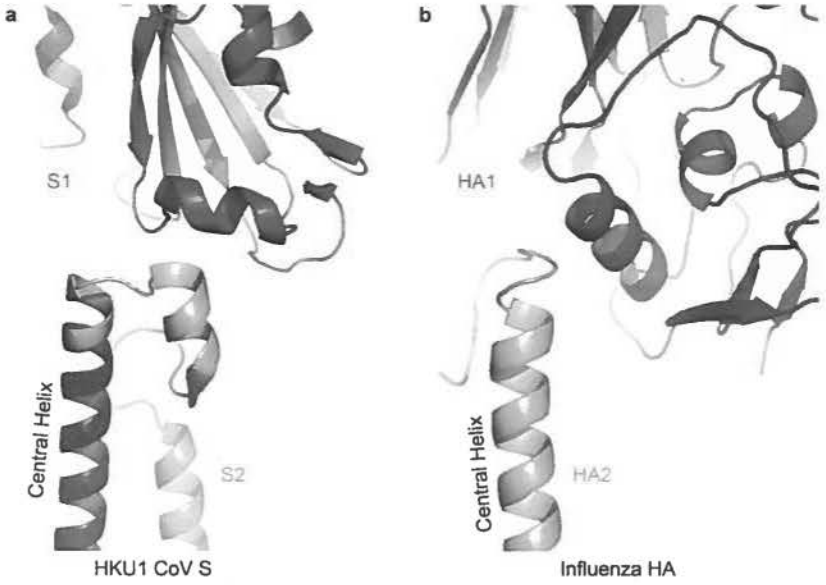
Extended Data Figure 3 | Putative glycan binding site of the HKU1 S NTD. The bovine coronavirus (BCoV) S NTD structure from Peng et al.[11] (teal) is superposed onto the HKU1 S NTD (pink). Residue side-chains involved in the putative glycan binding site are shown as sticks, with oxygen atoms colored red and nitrogen atoms colored blue. Note that N198 (BCoV) and N188 (HKU1) are predicted *N*-linked glycosylation sites.



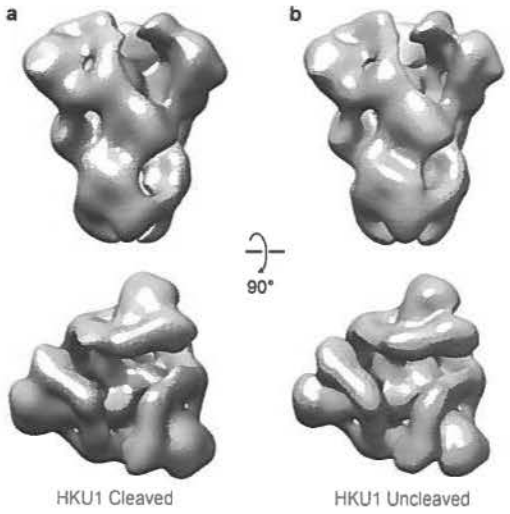
Extended Data Figure 4 | Betacoronavirus S proteins possess a conserved structural core in their C-terminal domains. a, The structurally divergent loop of the S1 CTD is poorly ordered distal to the core CTD domain. The conserved S1 CTD cores[13] of **b**, HKU1 Cov highlighted in the trimeric prefusion S, **c**, HKU1 CoV as an isolated domain, **d**, MERS CoV[15] and **e**, SARS CoV[14] are colored according to secondary structure (β-sheets: pink, α-helices: blue, lacking regular secondary structure: gray) and the insert which differs amongst coronaviruses is colored yellow. Atoms participating in quaternary interactions with other HKU1 S protomer CTDs are shown in green surface in (**c**). **f**, The positions of these interacting atoms are mapped on to the conserved core topology. The sheet and helix nomenclature is taken from reference[13].



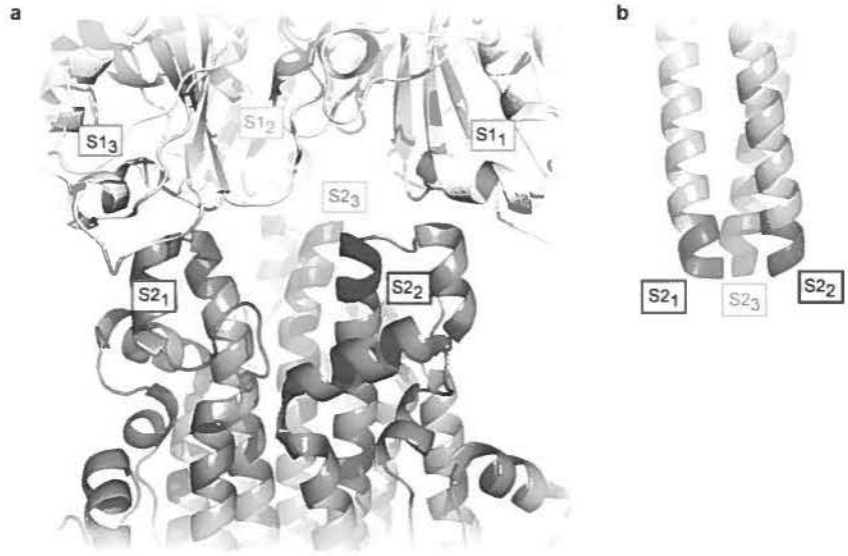
Extended Data Figure 5 | S1 sits atop an adjacent protomer's S2. a, The HKU1 S1 subunits are rotated about the trimeric 3-fold axis relative to their corresponding S2 subunits such that the S1 CTD from one protomer caps the S2 central helix from an adjacent protomer (CTD_1, blue, capping $S2_2$, red). The third protomer of the trimer has been omitted for clarity. **b,** The S2 N-terminal strand is connected to the remainder of the S2 subunit via a loop and an α-helix (dotted lines). These regions of the EM density are of insufficient quality to confidently build this protein region but enable interpretation of connectivity.



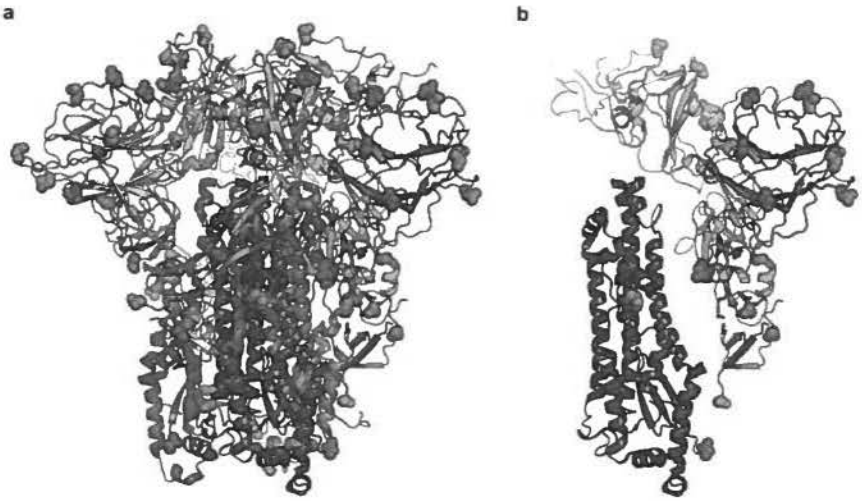
Extended Data Figure 6 | The central helix of HKU1 S2 is capped by the receptor-binding subunit. a, HKU1 CoV S1 CTD (blue) uses a short helix to cap the central helix (dark red) and a portion of HR1 (pink). **b**, The influenza hemagglutinin (HA) HA2 central helix is also capped by a helix in HA1 (blue)[18,30].



Extended Data Figure 7 | Cleavage at the S1/S2 junction does not induce large conformational changes in HKU1 spike. a, HKU1 spike 1–1249 with an attached foldon domain and wild-type furin-cleavage site was reconstructed using negative-stain electron microscopy. **b**, HKU1 spike 1–1276 with an attached foldon and a mutated furin-cleavage site reconstructed using negative-stain electron microscopy. Side and top views are shown.



Extended Data Figure 8 | The central helices of the prefusion S2 subunit splay open. a, In the prefusion HKU1 S protein, the tops of the central S2 helices (blue, red, green) are splayed outwards from the three-fold axis and capped by the S1 CTDs (white). The S1 NTD, SD-1 and SD-2 have been omitted for clarity. **b**, In the postfusion six-helix-bundle structure of SARS S[26], the corresponding helical regions form a well-packed three-helix bundle.



Extended Data Figure 9 | HKU1 S glycosylation. a, Sites of *N*-linked glycosylation on the HKU1 S trimer and **b**, a single monomer. Of the 30 potential *N*-linked glycosylation sites in a single protomer, the asparagine residues are observed for 21 sites and of these a small portion of density in the EM map is observed for 10 sites corresponding to the EndoH-trimmed sugars. Asparagines where glycan density is observed are shown as magenta spheres. Asparagines lacking glycan density are shown in green.

Abstract

Structure-based design of a prefusion-stabilized CoV S protein

Nianshuang Wang, Robert N. Kirchdoerfer, Christopher A. Cottrell, Jesper Pallesen, Hadi M. Yassine, Hannah L. Turner, Kizzmekia S. Corbett, Barney S. Graham, Andrew B. Ward& Jason S. McLellan

Coronaviruses (CoV) are large enveloped RNA viruses that usually cause mild illnesses in animals and humans. However, SARS-CoV and MERS-CoV can cause severe acute respiratory diseases that present severe challenges to global public health. The CoV spike (S) protein, a large trimeric protein on the surface of the virion, binds the host receptor and mediates fusion of the viral and host cell membranes by undergoing a large conformational change from the prefusion state. The S protein is the main target for protective antibody responses and also a critical candidate for vaccine design. However, the low expression level and instability of prefusion S proteins have hindered their development as vaccine immunogens. To address this issue, we recently determined the cryo-EM structure of hCoV-HKU1, and used this structural information to rationally design mutations that stabilize S proteins in the prefusion conformation. The same mutations stabilized SARS-CoV, MERS-CoV and hCoV-HKU1 S protein, boosted the expression of trimeric S protein by 10~100 fold and generated more homogeneous protein samples. The stabilized MERS S protein can also induce high neutralizing antibody titers in mice, indicating that it is a promising candidate for immunogen design. Collectively, these results demonstrate a universal stabilizing method for β-coronavirus prefusion S proteins, and additionally provide insights into the structure and function of CoV S proteins.

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An EIR should be completed for each discovery or invention that is a) a novel innovation, b) a new or improved method or process; **o**r c) believed to have potential commercial value (e.g. a new reagent, unique antibody, vaccine, medical device, or therapeutic compound); **or** d) a request from a commercial organization for use or resale of the new discovery or innovation.

If you are employed by PHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

COMPLETION OF THE EIR

1. This is the WORD 2007 formatted version
   http://www.ott.nih.gov/forms_model_agreements/forms_model_agreements.aspx
   - complete the form by filling in the shaded fields. For "check boxes" insert "X".
   - once completed, print the EIR and have each contributor sign their Contributor Information Sheet
   - questions regarding the completion of the EIR should be referred to your NIH/FDA Technology Development Coordinator (TDC)
   - email the completed electronic EIR template and any documents listed in questions 2.e., 5 or 6 to your NIH/FDA Technology Development Coordinator (TDC); and
   - Submit the signed EIR to your NIH/FDA Technology Development Coordinator (TDC)

2. The TDC will then forward the completed electronic report and the printed, signed EIR to the Office of Technology Transfer (OTT). If your IC in conjunction with the OTT decides not to file a patent application on your invention you may contact your Technology Development Coordinator (TDC) to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

   Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

   General questions regarding the form may be directed to the NIH Office of Technology Transfer (OTT). It is suggested, particularly if you leave government service and are receiving royalties, that you keep the Office of Financial Management apprised of changes in your official address.

Thank you for your cooperation

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Employee Discovery and Invention Report (EIR)

IC Ref: Date Received OTT Reference # Date Rcd

First Name	Middle Name	Last Name	Email
Barney	Scoh	Graham	(b) (6)

1. **Title of Discovery:** (In 200 characters or less provide a title that is sufficiently descriptive to identify the discovery.)

(b) (4) monoclonal antibodies against Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

2. **Please provide a clear and concise description of your discovery.** Describe in terms that an individual in another field would understand.

a. Provide background information related to the discovery.

There are no prophylactic or therapeutic countermeasures against SARS-CoV. Monoclonal antibodies (mAbs) represent a potential therapeutic solution for SARS-CoV-infected individuals. (b) (4)

b. Describe how the discovery will be used and what product(s) could be made.

These mAbs can be used as prophylactic or therapeutic countermeasures, as well as for diagnostic and specialized research reagents including (b) (4)

c. Describe the unique features and advantages of this discovery that distinguishes it over the problems or limitations associated with the current science or existing products.

(b) (4) mAbs against SARS-CoV were identified targeting (b) (4) (b) (4) Several mAbs have (b) (4) capacity. The combination of two or more mAbs of different specificities would likely be useful in preventing the possibility of viral escape.

d. *(if applicable)* If the discovery is or relates to a composition, describe the composition by providing sequence structures of amino acids or nucleic acids, provide chemical formulas for chemical compounds; if the discovery is a method, outline the particular steps of the method.

Antibody sequences are provided

e. Please list the most pertinent articles, presentations, other public disclosures, relevant patents or patent applications made by others that are related to your discovery?

These antibodies have not been publicly disclosed.

Attach copies of the publications cited; copies of U.S. Patents or published patent applications are not required.

3.

a. **Current Stage of Development** (*Please insert "X" for all the following states of development that currently best represent your discovery at this time)*

	Conceptual		Design		Working		Model/Prototype
x	In-vitro testing		In-vivo testing. Specify model				
	Model Organism Created: Specify						
	FDA submissions		IND		NCE/NME		IDE
	Clinical Trial Phase				Other		
x	Research Material(s)						
	Software (beta or later) [*please complete Attachment 2]*						

b. Future Development Activities

1. What activities related to this discovery will you be undertaking over the next 2 years?

(b) (4)

2. Would you consider working with a CRADA partner to further advance this discovery? If Yes,

Yes

4. Potential Commercial Applications

a. Does this disclosure include research material(s) that could be made available for licensing? If Yes, complete Attachment 1.

b. Is this EIR being submitted based on an outside party's request for licensing a material?, If Yes, provide the name of the company's contact person and telephone number or email.

Yes

c. Identify any company(s) or non-profit organizations that are conducting similar research.

(b) (4)

d. Have you been contacted by or are you in discussions with anyone interested in collaborating with you to develop this discovery?

Yes, (b) (4)

Thinking creatively, answer the following:

e. Describe how the discovery or material may enhance the public health, i.e. therapeutic, diagnostic, drug screening, assay, research tool, medical imaging software, or other software.

These mAbs can be used as prophylactic or therapeutic countermeasures, as well as diagnostic and specialized research reagents including antigenic characterization of vaccines

5. Public Disclosures/Presentations (Past and Future).

a. List any disclosure of this discovery, including partial disclosures, made by you or anyone identified in 7.b. Examples of disclosures include, but are not limited to the following: abstracts, posters, oral presentations/seminars, NIH Annual Reports, theses/dissertations, grant applications, reports, journal publications, website postings, phone calls, emails, demonstrations, sales catalogs, news releases, and any public use, sale, or offer for sale of the technology.

No publications but the VRC is currently sending these antibodies to potential collaborators under MTAs and RCAs.

b. Has a manuscript(s) been prepared (or in the process of being prepared) or submitted for review? If Yes (**attach a copy, *early first drafts are encouraged and will be kept confidential***)

i. Primary manuscript: -specify the (exact or expected) date of submission	
-identify the journal's name; publisher's telephone number or email (if known)	
-identify the date of publication (if known)	
ii. Additional manuscripts (draft and submitted) information:	

c. Has an abstract or poster been prepared or is now in preparation for a future presentation or publication? if Yes **attach a copy, (*early first drafts are encouraged and will be kept confidential*)**. For each presentation or publication: please identify, as appropriate, the following:

No.	Conference Symposium	Meeting Date (mm/dd/yy)	Date Abstract / Poster was submitted? (mm/dd/yy)	Abstract / Poster pre-published *online* or in *catalogue*?	Conference Organizer and Contact Information (Tab out of last cell to add additional row)
1.					
2.					

d. Is anyone outside of the Public Health Service (NIH, FDA, and CDC) aware of your discovery? If Yes, please identify them, the date, and describe the circumstances. (e.g. formal or informal communications with colleagues, collaborators)

Yes, (b) (4).. We have or are estabilishing RCAs with these groups, and have told them about the existance of these antibodies, but no sequences were shared. Sequences were shared with academic collaborators at Kansas University and Jason McLellan at UT Austin through RCAs.

6. Collaborations, Material Transfers, Confidential Disclosure Agreements

The following questions are designed to answer whether any part of the research resulting in this discovery involved collaborations outside of your laboratory, used materials/software/equipment obtained from an another party, and/or included outside funding. (Consult with your laboratory chief and/or your TDC if there is a question.)

a. CRADA: Is the subject matter of your disclosure related to a PHS Cooperative Research and Development Agreement (CRADA or M-CRADA) involving you or your laboratory or IC?,
If Yes, please identify the collaborator, the NIH principle investigator, CRADA title and the Institute's CRADA reference number:
No

b. Collaborations: Is the subject matter based on research collaborations other than a CRADA? If Yes, please identify your collaborator and the name of their organization.
No

c Outside Materials/Information Transfer: Is there subject matter in this discovery based on proprietary materials or confidential information obtained from an outside party or organization?,
If Yes, please identify the third party contributor, their email or telephone number, and the organization's name.
No

Please provide a copy of any document that records this transfer. This may include an email, Material Transfer Agreement, Confidential Disclosure Agreement, Clinical Trial Agreement, or Research Collaboration Agreement.

d. Non-NIH awards: Did this discovery arise based on non-NIH support? (e.g. grants, fellowships) If Yes, please identify
No

e. Human Subjects: Does this discovery rely upon materials or information obtained from human subjects or from data involving human subjects as defined in and regulated under 45 CFR Part 46? , If Yes, please provide the Protocol title, Institutional Review Board (IRB) protocol approval number and date or the OHSR exemption number, name ***or*** explain fully.
VRC 200

7. Identification of Contributors

a. Identify the individuals who *could* merit co-authorship credit for any associated publication.

b. List below the names and organizations of **all people** who participated in conceiving or continued development of the discovery/invention. Examples include those who made intellectual, theoretical, or innovative contribution to the discovery. In the case of software, those individuals who were involved in creating program code, manuals, flowcharts or any related items.
NOTICE: This may be fewer individuals than named in 7.a. above. *Please be aware that inventorship is strictly defined in patent law. Accordingly, contributors you list in this section will be named on patent applications resulting from this EIR only if their contributions meet this legal standard.* A co-author may or may not qualify based on the particular facts; if you have any questions, contact your TDC.

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

1. Submitting Contributor :	Barney Graham	: Org	VRC, NIAID

2. Co-Contributor :	John Moscola	: Org	VRC, NIAID
3. Co-Contributor :	Lingshu Wang	: Org	VRC, NIAID
4. Co-Contributor :	Yi Zhang	: Org	VRC, NIAID

6. Enter additional Co-Contributor's names and organizations as necessary.

Wing-pui Kong, VRC, NIAID, Kizzmekia Corbett, VRC, NIAID, Olu Abiona, VRC, NIAID

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The form may be downloaded at: http://www.ott.nih.gov/docs/EIR-Additional-Contributor.doc

Contributor Information begins on next page

Employee Discovery and Invention Report (EIR)
Contributor Information

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Contributor 1

Name

♦First	Yi	♦Middle		♦Last	Zhang	Suffix	
Degree	B. S	♦Citizenship	(b) (6)				
♦Employee Identification No.:	2000750029	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

Designed experiments, isolated and characterized monoclonal antibodies

Current Organization Information:

♦Organization Name	NIAID,NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Biologist						
♦Office Address	Room 4606B, Building40, 40 Convent Drive						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	US
Phone		Email					

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section: Contract employer: Kelly Government

Contributor: I have read and understand the information submitted in the EIR.

(b) (6) 3/19/2020

Signature **Date**

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Contributor 1

Name

♦First	John	♦Middle	R	♦Last	Mascola	Suffix	
Degree	MD	♦Citizenship	(b) (6)				
♦Employee Identification No..	001-1058-566			♦Associated project NIH Z01 Project Number(s)			

♦**Describe this individual's contribution to the discovery.**

Oversaw laboratory activities leading to isolation of the antibodies. Oversaw laboratory activities to develop the SARS and CoV-2 neutralization assays.

Current Organization Information:

♦Organization Name	Vaccine Research Center, NIAID						
Division/Branch/Laboratory	Virology Laboratory						
♦Title	Director, Vaccine Research Center						
♦Office Address	40 Convent Drive, Bldg 40, Rm 4504						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature **Date** **March 18, 2020**

Employee Discovery and Invention Report (EIR)
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Contributor 1

Name

♦First	Olubukola	♦Middle	Mary Abisola	♦Last	Abiona	Suffix	
Degree	Bachelor of Science	♦Citizenship	(b) (6)				
♦Employee Identification No.:	200-2358-423			♦Associated project NIH Z01 Project Number(s)			

♦**Describe this individual's contribution to the discovery.**

Construct design, testing & characterization

Current Organization Information:

♦Organization Name	National Institute of Health						
Division/Branch/Laboratory	NIAID-VRC						
♦Title	Post-baccalaureate IRTA Fellow						
♦Office Address	40 Convent Drive, Rm 2608						
♦City	(b) (6)	♦State	Maryland	♦Zip code	(b) (6)	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	Maryland	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar	X	IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature 03/18/2020 **Date**

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Contributor 1

Name

♦First	Lingshu	♦Middle		♦Last	Wang	Suffix	
Degree	Ph.D.	♦Citizenship	(b) (6)				
♦Employee Identification No.	0013473896	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

Designed experiments, isolated and characterized monoclonal antibodies

Current Organization Information:

♦Organization Name	NIAID, NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Staff Scientist						
♦Office Address	Room 4608B, Building 40, 40 Convent Drive						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	US
Phone		Email					

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6) 3/19/2020

Signature **Date**

Employee Discovery and Invention Report (EIR)
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Contributor 1

Name

♦First	Wing Pui	♦Middle		♦Last	Kong	Suffix	
Degree	Ph.D.	♦Citizenship	(b) (6)				
♦Employee Identification No..	0010151956	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

Headed the design and performing animal studies of SARS-CoV immunogens immunization to isolate SARS-CoV- specific monoclonal antibodies.

Current Organization Information:

♦Organization Name	NIAID, NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Chief of Virology Core						
♦Office Address	40 Convent Drive, Rm 4608						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX	(b) (6)	Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

Home Information

♦City	(b) (6)	♦State	Maryland	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee – specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

3-19-2020

Employee Discovery and Invention Report (EIR)

Attachment 1

Supplemental Information Sheet for Research Materials

This attachment requests information regarding materials that may be available for potential licensing. The **Set 1** questions are general information regarding specific materials required to practice the invention or ancillary materials created during the course development that may be potentially licensed.

The **Set 2** questions SHOULD BE ANSWERED IF THIS EIR is being submitted based on an outside party's request for licensing a material.

Set 1. (not required if Set 2 Questions are completed) General material information.

a) Identify those material(s) made during course of research that are specifically required to practice this invention. Please identify each unique material(s) or chemical compound(s) developed that are related to this EIR.

b)Identify those material(s) made during course of research that may be available for licensing as a research material. Please identify each unique material(s) or chemical compound(s) developed that are related to this EIR.

c) Material Citation or other source:

d) Has material been deposited in the ATCC or similar repository? If Yes, please provide the repository and catalog reference number.

e) Were any of the materials necessary to use or make the Material acquired from someone outside NIH? If Yes and not already listed in Question 6c, please provide contact information and a copy of any document that records this transfer.

Set 2. If a prospective licensee is interested in a material answer the following questions.

Provide a summary regarding the approximate difficulty/time/cost/effort for your laboratory to provide the material to an outside for-profit requestor. Is it a limited resource?

Identify and describe the Material Type

Examples of potential material types:
Antibodies: monoclonal, polyclonal | **Cell Lines**: uninfected cells, infected cells, hybridomas
DNA/RNA: genetic clones, expression vectors, PCR reagents:
Proteins/Peptides: | **Purified Proteins**: synthetic peptides
Viruses: virus isolates, drug resistant, virus isolates, recombinant vaccinia
Opportunistic Infections: Candida, Cryptococcus, cryptosporidium, cytomegalovirus, mycobacterium, mycoplasma, pneumocystis, toxoplasma
Model Organisms (e.g. strain, species) | **Chemical Compounds**

Designation: (Laboratory nomenclature

Source of Material: (i.e. human, mouse, rat)

Reference Citation or other source

Has the material been deposited in the ATCC or similar repository? If Yes, please provide the repository and catalog reference number.

How can it be provided: (i.e. 2ml vial of frozen cells, plasmid

Current quantities available for distribution: (i.e. 10 vials)

Recommended propagation medium and growth characteristics: (i.e. expression level, titer, temperature, passages

Recommended freeze medium

Sterility: (i.e. negative for bacteria, fungi & mycoplasma

Morphology: (i.e. epithelial-like, lymphoblast-like, fibroblast like)

Recommended Storage: (i.e. liquid nitrogen)

Employee Discovery and Invention Report (EIR)

Attachment 2

Supplemental Information Sheet for Software

The purpose of this attachment is to provide information regarding evaluation of software for potential licensing.
The following questions should be answered as completely as possible.

1. Does this software contain code obtained from a third-party or covered by any Open Source License (e.g., collaborator, under a software agreement, a vendor, purchased etc.)? If Yes, please explain

2. Has this software been previously copyrighted? If Yes, then by whom?

3. Did you use outsiders to beta-test code? If Yes, was this done under an agreement?

4. How would the lab generally classify the use of this software (e.g., imaging, array analysis, mapping etc.)?

5. From the lab's perspective, what would be the preferred way of distributing this software?

6. That are the operating system requirements to run this software?

7. In which computer language is the software code written?

8. What stage of development is the software? Select one of the following:

	Ready to use by anyone
	Usable with some effort or assistance
	Needs substantial further development

9. Is the lab willing to release the source code?

10. Is the lab willing to prepare a demonstration version of the software to give to prospective licensees?

11. Were the current or prior versions distributed? If Yes, explain and supply date of distribution and any distribution agreement (if any).

12. Was a government contractor involved in the writing or development of any of the code? If yes, identify the individual.

Employee Discovery and Invention Report (EIR)
Institute/Center Administration Section
(To be completed by the IC Technology Transfer Office.)

Lead IC sponsoring this invention	NIAID	Division/Lab/Bran (if applicable)	VRC
Identify any other ICs (if necessary, include Division/Lab/Branch	N/A		
Confirm the scientist who will serve as the key point of contact (Scientific Point of Contact (SPC)) to receive and respond to patent correspondence. This may be different from the person identified as the Submitting Contributor.	Graham		

IC is requesting OTT to perform the following action regarding this EIR. Only 1 of the following option boxes should be checked.

Do Not File

	Do not file; EIR is being submitted due to a request for material(s) licensing. Requester's contact information is attached
	Do not file; market as a research material
	Do not file a patent application based on other patent and/or policy reasons

Evaluation/Searching

	Request OTT evaluation and recommendation, Outside search/opinion are authorized, if needed
	Request OTT evaluation and recommendation, Outside search/opinion is not authorized

Patent Application Filing

x	Emergency Patent Filing - File immediate provisional patent application based on an imminent publication. Authorization for filing is provided. In the "Additional Information or Instructions" box enter the date, location of public release and any other instructions
	File Patent: File a fully enabled provisional patent application
	File Patent/Patent Opinion: File a fully enabled provisional patent application and conduct a patentability opinion/assessment immediately after the provisional patent application has been filed
	Patent Opinion/File Patent: Conduct a patentability opinion/assessment (Completed by contract law firm). If the patentability opinion/assessment is positive, proceed with the filing a fully enabled provisional patent application
	Third Party Filing Patent Lead: Third party has already filed or will be doing the filing. Please provide 3rd Party contact information. Application number(s), filing date(s), and copy(ies) of application(s) should be provided as well as filing receipts or paperwork identifying the inventors as filed
	Other Filing Instructions

Additional Information or Instructions

	N/A

Is the IC submitting this EIR represented by a Service Center for this EIR? If Yes, please specify to whom the patent recommendations and patent correspondence are to be forwarded.

[ ] Submitting IC TDC only [ ] Service Center only [ ] Both the IC and Service Center

If documents are to be sent to the service center specific in "Additional Information or Instructions" box the individual to whom all patent recommendations and patent correspondence are to be directed.

The TDC, IC delegate, or Service Center Representative confirms receiving the EIR and acknowledges a complete EIR packet is being forwarded to OTT.

Name	Vincent Feliccia, JD, PhD	Date	
Title	Branch Chief, TTIPO, NIAID		

Signature: Vincent L. Feliccia -S Digitally signed by Vincent L. Feliccia -S Date: 2020.03.20 13:07:16 -04'00'

Authorized IC official for expenditure of IC funds for patent related expenses or attach authorization memo.

Name	Michael R. Mowatt, PhD	Date	
Title	Director, TTIPO, NIAID		

Signature: Michael R. Mowatt -S Digitally signed by Michael R. Mowatt -S Date: 2020.03.20 17:04:06 -04'00'

A complete EIR packet will be forwarded to the Director, DTDT, which comprises a single, non-stapled EIR packet containing the signed EIR containing all documents such as manuscripts, presentation, articles and citations referred to in the EIR, as well as any related IC reviews and authorization documents. The electronic version containing all the above information should be forwarded to OTT at ottfileroom@mail.nih.gov

Employee Discovery and Invention Report (EIR)

1. **Title of Discovery:**

   Stabilized Wuhan coronavirus constructs for vaccination

2. **Brief Description of Discovery** *(in less than 200 words)*:

   Stabilized coronavirus proteins. This invention provides amino acid mutations to increase the stability of a prefusion conformation of Wuhan coronavirus proteins including but not limited to: (b) (4) with the goal of improving immunogenicity of coronavirus vaccines

   Your discovery should be documented in your lab records. Please ensure that you maintain signed, witnessed, and dated lab records, but do not enclose them. E.g. see https://ltc.nci.nih.gov/pdfs/brochures/Keeping_Lab_Records.pdf

3 **Expanded Description of Discovery** *(Please attach a description of the technology in MS Word specifically describing what is new about the discovery including any sequences, compositions, structures, formulas, steps of a method etc. The description may be by reference to a separate document such as a copy of a report, preprint, manuscript or preliminary results and the like. Please include a MS Word copy if possible).* If this is a modification or improvement to an existing work or incorporates elements (software, confidential information, material) that are not original to you or your lab, please identify that work and any original creators.

3. **Technology Significance**
   Describe the unique advantages of this discovery over the current science/technology:

   Stabilized proteins will allow for improved immunogenicity of vaccines against the newly emergent betacoronaviruses, 2019-nCoV/Wuhan coronavirus as well as possibly more broad protection against other betacoronaviruses.

4. **Was this discovery made as part of a collaboration, contract, CRADA, or grant with an outside entity?**

(indicate yes or no)						
	x	Yes		No		Don't Know
Is there a written agreement with the collaborator?	x	Yes		No		Don't Know
Was the collaboration part of a CRADA?		Yes	x	No		Don't Know
Did the collaborator provide materials?		Yes	x	No		Don't Know
Did the discovery involve human materials or subjects?		Yes	x	No		Don't Know

Name of Collaborator	Material Provided (if any)	Agreement Type/ # (if known)	Date
Jason McClellan, University of Texas at Austin (RCA)			

5. **Publication or Other Disclosure of Discovery**
   Was the discovery: submitted to a journal, published, presented orally or as a poster, listed on a website, discussed with non-NIH personnel, or otherwise disclosed? If yes, describe (e.g. title, journal, public URL, hand-outs, location) and provide a date for each:

   Invention is related to previously-filed patent applications in the (b) (4) technology family

   Are any future disclosures planned? If yes, provide estimated date(s):

   Provide 1) information/ attach PDFs regarding any disclosures above, and 2) citations to any work others have done in this specific area (e.g. scientific papers, patent /application numbers, public access web sites) and, if available, copies of cited documents:

   Citations:

6. **Technology Stage** *(Choose all that apply)*

x Concept	x Prototype	Modification	*In vitro*	*In vivo*	Clinical	Final Product	Research Use

7. **Future Research Plans** Description of any additional research that is needed in order to complete development and testing of the invention (use an additional page if necessary):

   Generate *in vitro* and *in vivo* data on the constructs that have been designed

(a) Is this research presently being undertaken?	x	Yes		No	If yes, list any outside collaborator:	McLellan
(b) Actively pursued by other PHS staff?		Yes	x		If yes, identify staff:	
(c) Actively undertaken by a corporate partner?	x	Yes		No	If yes, identify corporate partner:	(b) (4) are engaged under RCAs
(d) Do you want to seek corporate partnership?		Yes		No		

(e) Do you think this technology could form the basis of a "start-up" company? Yes No x Don't Know

9. **Commercial Potential** *(be creative)* Suggest any products, processes ,services you could envision resulting from this invention, and whether they can be developed in the near term (less than two years) or long term:

   Incorporated in optimized vaccines against coronaviruses

10. **Competition and Potential Users and Manufacturers**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

(a) Describe technologies, products, processes or services currently on the market of which you are aware that accomplish the purpose of this invention; or that are similar to this technology but used for a different purpose: None known

(b) List any companies you believe may be interested in this technology: Companies involved in development of vaccines

(c) If you have a contact at any of these companies, please provide name, email and phone numbers for each, if available:

List the names and organizations of **all people** who participated in conceiving or continued development of the discovery/invention. Examples include those who made intellectual, theoretical, or innovative contribution to the discovery. In the case of software, those individuals who were involved in creating program code, manuals, flowcharts or any related items.

1. Submitting Contributor	Barney Graham ✓	Organization:	VRC
2. Co-Contributor	Jason McLellan	Organization:	UT-Austin
3. Co-Contributor	Kizzmekia Corbett ✓	Organization:	VRC
4. Co-Contributor	Olu Abiona ✓	Organization:	VRC

Enter additional Co-Contributor's names and organizations as necessary:
Geoffrey Hutchinson (VRC); Daniel Wrapp (UT-A); Nian Shuang (UT-A)

An Additional Contributor Information document (to follow) is to be completed for each contributor listed above. If required, extra forms may be downloaded at: http://www.ott.nih.gov/sites/default/files/documents/docs/eir-additional-contributor.docx

In addition to the above names, identify any individuals who could merit authorship credit of any associated publication:

NOTICE: There may be fewer individuals listed as contributors than named as coauthors. *Please be aware that inventorship is strictly defined in patent law. Accordingly, contributors you list in this section will be named on patent applications resulting from this EIR only if their contributions meet this legal standard.* A co-author may or may not qualify based on the particular facts; if you have any questions, contact your .

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Detailed Contributor Information begins on next page

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Contributor 1

Name

♦First	Geoffrey	♦Middle	Bain	♦Last	Hutchinson	Suffix	
Degree		♦Citizenship	(b) (6)				
♦Employee Identification No.	2002248736			♦Associated project NIH Z01 Project Number(s)			

♦**Describe this individual's contribution to the discovery**.

Construct design, testing, characterization

Current Organization Information:

♦Organization Name	NIH, NIAID, Vaccine Research Center						
Division/Branch/Laboratory	Viral Pathogenesis Lab						
♦Title	Postbac IRTA						
♦Office Address	Vaccine Research Center, building 40, Convent Dr. #2608						
♦City	Bethesda	♦State	Maryland	♦Zip code	20892	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No.

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls uncer one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		CRDF Fellow		Non-CRDF
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar	X	IRTA Internship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog.		National Research Council Award		Howard Hughes Medical Institute		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature

2/9/2020

Date

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Barney	♦Middle	Scott	♦Last	Graham	Suffix	Dr.
Degree	MD, PhD	♦Citizenship	(b) (6)				
♦Employee Identification No.:	001-0633-378	♦Associated project NIH Z01 Project Number(s)					

♦Describe this individual's contribution to the discovery.

Conceived the approach and supervised the work to develop the candidate vaccine

Current Organization Information:

♦Organization Name	Vaccine Research Center						
Division/Branch/Laboratory	NIAID/VRC/VPL						
♦Title	Deputy Director						
♦Office Address	40 Convent Dr. Room 2504 MSC 3017						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX	(b) (6)	Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email					

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature **Date** 5 Feb 2020

Employee Discovery and Invention Report (EIR)
Additional Contributor Information

The Additional Contributor Information document should be used when an EIR has already been completed and there are more than four (4) Contributors identified in the EIR. It is to be completed by any individual whose name was listed as a Contributor, but their Contributor information was not provided in EIR Question 7.

If you are employed by PHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

COMPLETION OF THE CONTRIBUTOR INFORMATION PAGE

1. Review the EIR
   - download the fill able electronic Additional Contributor Information Page WORD template form.
   - complete the Contributor Information Page found on the next page by filling in the shaded fields. For "check boxes" insert "X".
   - questions regarding this EIR should be referred either to NIH/FDA Technology Development Coordinator (TDC)
   - print and sign the form
   - email the completed Contributor Information Page to NIH/FDA Technology Development Coordinator (TDC); and
   - submit the printed and signed EIR to the Agency's TDC.

2. Upon receipt, the TDC will forward your information as part of the completed electronic EIR report and the printed, signed EIR to the Office of Technology Transfer (OTT). If your IC in conjunction with the OTT decides not to file a patent application on your invention you may contact your Technology Development Coordinator (TDC) to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

   Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

   General questions regarding the form may be directed to the NIH Office of Technology Transfer (OTT). It is suggested, particularly if you leave government service and are receiving royalties, that you keep the Office of Financial Management apprised of changes in your official address.

Thank you for your cooperation

Privacy Act Notice: The PHS is collecting this information under authority of 45 CFR Part 7 "Employee Inventions". The information will be maintained as a part of the System of Records: 09-25-0168, "Invention, Patent and Licensing Documents." Provision of this information is mandatory and will be used as the initial step toward obtaining patent protection of inventions submitted by PHS employees, granting licenses to PHS inventions, administering and providing royalty payments to PHS inventors, and the intended "routine uses" of the information. Failure to provide complete information may adversely affect the Government's rights to future patent applications and licensing agreements.

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, ecuipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Olubukola	♦Middle	Mary Abisola	♦Last	Abiona	Suffix	
Degree	Bachelor of Science	♦Citizenship	(b) (6)				
♦Employee Identification No.	200-2358-423	♦Associated project NIH ZO1 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

Construct Design, Testing, & Characerization

Current Organization Information:

♦Organization Name	National Institutes of Health						
Division/Branch/Laboratory	NIAID-VRC						
♦Title	Post-baccalaureate IRTA Fellow						
♦Office Address	40 Convent Drive, Rm 2608						
♦City	Bethesda	♦State	Maryland	♦Zip code	20892	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	(b) (6)

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	Maryland	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar	X	IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research in Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature

02/04/2020

Date

5

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Kizzmekia	♦Middle	Shanta	♦Last	Corbett	Suffix	
Degree	PhD	♦Citizenship	(b) (6)				
♦Employee Identification No.:				♦Associated project NIH Z01 Project Number(s)			

♦**Describe this individual's contribution to the discovery.**

Sequence Design

Current Organization Information:

♦Organization Name	NIH NIAID						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Research Fellow						
♦Office Address	40 Convent Drive, Room 2608						
♦City	Bethesda	♦State	MD	♦Zip code	20982	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX	(b) (6)	Other contact number. (optional)	(b) (6)

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	Maryland	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program	X	Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Employee Discovery and Invention Report (EIR)
Contributor Information

(b) (6)

[redacted]

Signature **Date** February 4, 2020

Employee Discovery and Invention Report (EIR)
Information

♦ Indicates a required field

Information about this Form

Reporting an invention is required as part of your Government service, and supports the mission of your IC and the NIH in advancing public health. An EIR should be completed for each discovery or invention* that is:

a) An innovation;
b) A new or improved method or process;
c) Believed to have potential commercial value (e.g. a new reagent, unique antibody, vaccine, medical device, or therapeutic compound); **or**
d) Requested from a commercial organization for use or resale.

If you are employed by HHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

IC means a Public Health Service (PHS) Contributor's Institute, Center, or Office (includes NIH, FDA and CDC).

COMPLETION OF THE EIR

1. Complete the form by filling in the shaded fields. For "check boxes" insert "X";
2. Once completed, have each contributor sign their Contributor Information Sheet;
3. Questions regarding the completion of the EIR should be referred to your TDC;
4. Email the completed electronic EIR template and any related documents to your TDC; and
5. After review by your TDC, email a signed copy of the final EIR to your TDC.
6. The TDC will then forward the completed and signed EIR to the Office of Technology Transfer (OTT). If your IC in decides not to file a patent application on your invention you may contact your TDC to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

General questions regarding the form may be directed to your TDC or the NIH Office of Technology Transfer (OTT). It is suggested, particularly if you leave government service and are receiving royalties, that you keep the Office of Financial Management apprised of changes in your official address.

Thank you for your contribution toward improving public health!

Privacy Act Notice: HHS is collecting this information under authority of 45 CFR Part 7 "Employee Inventions". The information will be maintained as a part of the System of Records: 09-25-0168, "Invention, Patent and Licensing Documents." Provision of this information is mandatory and will be used as the initial step toward obtaining patent protection of inventions submitted by HHS employees, granting licenses to HHS inventions, administering and providing royalty payments to HHS inventors, and the intended "routine uses" of the information. Failure to provide complete information may adversely affect the Government's rights to future patent applications and licensing agreements.

***What is the difference between a discovery and an invention?**

Discovery

- Elucidating something that already exists
- Embodied in Nature
- Discovery involves describing something
- Product of Nature
- e.g. A botanist discovers a new plant species on an island

Invention

- An innovation that did not previously exist
- Embodied in human-made artifact
- Always involves creating something
- Produced through human thought
- A botanist invents a new topical antibiotic formulation using the plant oils

Employee Discovery and Invention Report (EIR)
Administration Section
(To be completed by the IC Technology Transfer Office)

♦ Indicates a required field

Lead IC sponsoring this invention: NIAID Division/Lab/Branch VRC

Identify any other ICs (if necessary, include Division/Lab/Branch N/A

Confirm the scientist who will serve as the key point of contact (Scientific Point of Contact or SPC) to receive and respond to patent correspondence. This may be different from the person identified as the Submitting Contributor. Graham

IC CRADA Determination: (Answer required if the EIR is related to a CRADA)

The invention was conceived or first actually reduced to practice in the performance of the activities under the CRADA Research Plan during the term of the CRADA. Enter Y or N.

IC is requesting the following action regarding this EIR. *Only 1 of the following option boxes should be checked.*

Do Not File

Do not file; EIR is being submitted due to a request for material(s) licensing. Requester's contact information is attached

Do not file; market as a research material

Do not file a patent application based on other patent and/or policy reasons

Evaluation/Searching

Request evaluation and recommendation, Outside search/opinion are authorized, if needed

Request evaluation and recommendation, Outside search/opinion is not authorized

Patent Application Filing

x Emergency Patent Filing - File immeciate provisional patent application based on an imminent publication. Authorization for filing is provided. In the "Additional Information or Instructions" box enter the date, location of public release and any other instructions

File Patent: File a fully enabled provisional patent application

File Patent/Patent Opinion: File a fully enabled provisional patent application and conduct a patentability opinion/assessment immediately after the provisional patent application has been filed

Patent Opinion/File Patent: Conduct a patentability opinion/assessment (Completed by contract law firm). If the patentability opinion/assessment is positive, proceed with the filing a fully enabled provisional patent application

Third Party Filing Patent Lead: Third party has already filed or will be doing the filing. Please provide 3rd Party contact information. Application number(s), filing date(s), and copy(ies) of application(s) should be provided as well as filing receipts or paperwork identifying the inventors as filed

Other Filing Instructions

Additional Information or Instructions

Is the IC submitting the EIR represented by a Service Center? No

If yes, identify IC: N/A

All patent recommendations and patent correspondence will be directed the Lead IC's central email account.

Name of LPM designated for this EIR by IC: Amy Petrik

The TDC, IC delegate, or Service Center Representative confirms receiving the EIR and acknowledges a complete EIR packet is being forwarded to OTT.

Name Vincent Feliccia, JD, PhD Date 2/4/2020

Title Branch Chief, TTIPO, NIAID

Signature (b) (6)

Authorized IC official for expenditure of IC funds for patent related expenses or attach authorization memo.

Name Michael R. Mowatt, PhD Date 5 FEB 2020

Title NIAID TDC and Director, TTIPO, NIAID

Signature (b) (6)

An OCR'd PDF containing the signed EIR containing all documents such as manuscripts, presentations, articles and citations referred to in the EIR, as well as any related IC reviews and authorization documents should be forwarded to OTT at ottfileroom@mail.nih.gov. The email's subject line should include "New EIR for IC, (PI's Last Name), (IC Ref. #, if exists)." Original formatted documents, i.e. MS Word, PowerPoint, should be attached for EIRs with recommendations of "Evaluate", "File Patent", "Patent Opinion" or any combination of these.

Employee Discovery and Invention Report (EIR)
Appendix A - Supplemental Information Sheet for Research Materials

This attachment requests information regarding materials that may be available for potential licensing. The **Set 1** questions elicit general information regarding specific materials required to practice the invention or ancillary materials created during the course of development that may be potentially licensed. The **Set 2** questions SHOULD BE ANSWERED IF THIS EIR is being submitted based on an outside party's request for licensing a material.

Set 1. General material information. *(Not required if Set 2 Questions are completed)*

a) Identify those materials made during the course of research that are specifically required to practice this invention. Please identify each unique material or chemical compound developed that are related to this EIR.

Plasmids encoding the S-2P 2019-nCoV

b) Identify those material(s) made during the course of research that may be available for licensing as a research material. Please identify each unique material or chemical compound developed that are related to this EIR.

Plasmids encoding and protein embodying S-2P 2019-nCoV

c) Material citation or other source:

d) Has material been deposited in the ATCC or similar repository? If yes, please provide the repository and catalog reference number.

No

e) Were any of the materials necessary to use or make the Material acquired from someone outside NIH? If yes and not already listed in Question 5, please provide contact information and a copy of any document that records this transfer.

No

Set 2. If a prospective licensee is interested in a material answer the following questions.
Provide a summary regarding the approximate difficulty/time/cost/effort for your laboratory to provide the material to an outside for-profit requestor. Is it a limited resource?

Identify and describe the Material Type:

Examples of potential material types:

Antibodies: monoclonal, polyclonal
Cell Lines: uninfected cells, infected cells, hybridomas
DNA/RNA: genetic clones, expression vectors
PCR reagents
Proteins/Peptides
Purified Proteins
Viruses: virus isolates, drug resistant, virus isolates, recombinant vaccinia
Opportunistic Infections: (e.g. Candida, Cryptococcus, cryptosporidium, cytomegalovirus, mycobacterium, mycoplasma, pneumocystis, toxoplasma)
Model Organisms (e.g. strain, species)
Chemical Compounds

Designation: (Laboratory nomenclature)

Source of Material: (i.e. human, mouse, rat)

Reference Citation or other source:

Has the material been deposited in the ATCC or similar repository? If Yes, provide repository & catalog. ref.#s.

How can it be provided: (i.e. 2ml vial of frozen cells, plasmid)

Current quantities available for distribution: (i.e. 10 vials)

Recommended propagation medium & growth characteristics: (i.e. expression level, titer, temp., passages)

Recommended freeze medium:

Sterility: (i.e. negative for bacteria, fungi and mycoplasma)

Morphology: (i.e. epithelial-like, lymphoblast-like, fibroblast-like)

Recommended Storage: (i.e. liquid nitrogen)

Employee Discovery and Invention Report (EIR)
Appendix B - Supplemental Information Sheet for Software

The purpose of this attachment is to provide information regarding evaluation of software for potential licensing. The following questions should be answered as completely as possible.

1. Does this software contain code obtained from a third-party or covered by any Open Source License (e.g., collaborator, under a software agreement, a vendor, purchased etc.)? If Yes, please explain.

   N/A

2. Has this software been previously copyrighted? If yes, by whom?

3. Did you use outsiders to beta-test code? If yes, was this done under an agreement?

4. How would the lab generally classify the use of this software (e.g., imaging, array analysis, mapping etc.)?

5. From the lab's perspective, what would be the preferred way of distributing this software?

6. What are the operating system requirements to run this software?

7. In which computer language is the software code written?

8. What stage of development is the software? Select one of the following:

   Ready to use by anyone
   Useable with some effort or assistance
   Needs substantial further development

9. Is the lab willing to release the source code?

10. Is the lab willing to prepare a demonstration version of the software to give to prospective licensees?

11. Were the current or prior versions distributed? If yes, explain and supply date of distribution and any distribution agreement (if any).

12. Was a government contractor involved in the writing or development of any of the code? If yes, identify the individual.

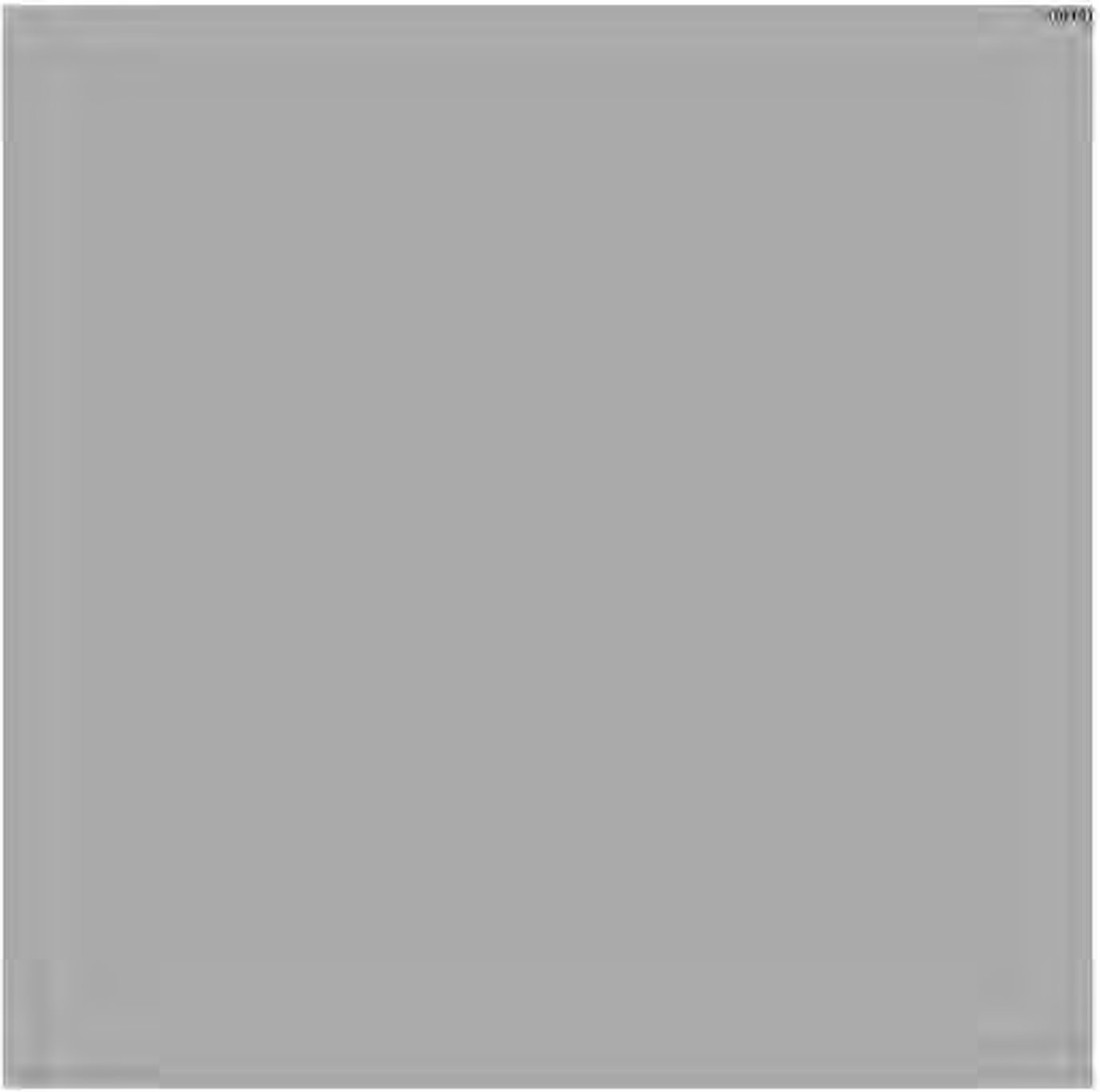
2019-nCoV Wuhan S-2P Sequence

(b) (4)

2019-nCoV Wuhan S Sequence (wild-type)

ACCESSION MN908947

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGTNGTK
RFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFLGVYYHKNNKSWME
SEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIYSKHTPINLVRDLPQGFSALEPLVDLPI
GINITRFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSFTV
EKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPT
KLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLK
PFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCV
NFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVN
CTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSYECDIPIGAGICASYQTQTNSPRRARSVASQSII
AYTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGI
AVEQDKNTQEVFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDCLGDIAAR
DLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAMQMAYRFNGIGVTQNVLYENQKLIA
NQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRL
QSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTA
PAICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNTVYDPLQPELDSFKEELDKY
FKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIKWPWYIWLGFIAGLIAIVMVTI
MLCCMTSCCSCLKGCCSCGSCCKFDEDDSEPVLKGVKLHYT



Employee Discovery and Invention Report (EIR)

1. **Title of Discovery:**

Anti-Coronavirus Antibodies and Methods of Use

2. **Brief Description of Discovery** *(in less than 200 words)*:

Monoclonal antibodies against 2019 nCoV for use in the diagnosis, monitoring, and treatment of SARS-Co-V and SARS-CoV-2-linked diseases such as COVID-19. The filing (attached) contains a large library of (b) (4) antibodies against (b) (4) of coronaviruses. (b) (4)

Your discovery should be documented in your lab records. Please ensure that you maintain signed, witnessed, and dated lab records, but do not enclose them. E.g. see https://ttc.nci.nih.gov/pdfs/brochures/Keeping_Lab_Records.pdf

3 **Expanded Description of Discovery** *(Please attach a description of the technology in MS Word specifically describing what is new about the discovery including any sequences, compositions, structures, formulas, steps of a method etc. The description may be by reference to a separate document such as a copy of a report, preprint, manuscript or preliminary results and the like. Please include a MS Word copy if possible).* If this is a modification or improvement to an existing work or incorporates elements (software, confidential information, material) that are not original to you or your lab, please identify that work and any original creators.

3. **Technology Significance**

Describe the unique advantages of this discovery over the current science/technology:

Potential therapeutic, diagnostic and/or prevention against 2019 nCoV

4. **Was this discovery made as part of a collaboration, contract, CRADA, or grant with an outside entity?**

(indicate yes or no)						
	x	Yes		No		Don't Know
Is there a written agreement with the collaborator?	x	Yes		No		Don't Know
Was the collaboration part of a CRADA?		Yes	x	No		Don't Know
Did the collaborator provide materials?		Yes	x	No		Don't Know
Did the discovery involve human materials or subjects?	X	Yes		No		Don't Know

Name of Collaborator	Material Provided (if any)	Agreement Type/ # (if known)	Date
(b) (4)		RCA (b) (4)	March 11, 2019
(b) (4)			

5. **Publication or Other Disclosure of Discovery**

Was the discovery: submitted to a journal, published, presented orally or as a poster, listed on a website, discussed with non-NIH personnel, or otherwise disclosed? If yes, describe (e.g. title, journal, public URL, hand-outs, location) and provide a date for each:

Provisional patent filing (attached)

Are any future disclosures planned? If yes, provide estimated date(s):

Provide 1) information/ attach PDFs regarding any disclosures above, and 2) citations to any work others have done in this specific area (e.g. scientific papers, patent /application numbers, public access web sites) and, if available, copies of cited documents:

Citations:

6. **Technology Stage** *(Choose all that apply)*

	Concept	Prototype	Modification	x	*In vitro*	*In vivo*	Clinical	Final Product	Research Use

7. **Future Research Plans** Description of any additional research that is needed in order to complete development and testing of the invention (use an additional page if necessary):

(a) Is this research presently being undertaken?	x	Yes		No	If yes, list any outside collaborator:	
(b) Actively pursued by other PHS staff?		Yes	x	No	If yes, identify staff:	
(c) Actively undertaken by a corporate partner?		Yes	x	No	If yes, identify corporate partner:	
(d) Do you want to seek corporate partnership?		Yes	x	No		

(e) Do you think this technology could form the basis of a "start-up" company? Yes No Don't Know

9. **Commercial Potential** (*be creative*) Suggest any products, processes ,services you could envision resulting from this invention, and whether they can be developed in the near term (less than two years) or long term:

Potential therapeutic against CoV, including 2019 nCoV

10. **Competition and Potential Users and Manufacturers**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

(a) Describe technologies, products, processes or services currently on the market of which you are aware that accomplish the purpose of this invention; or that are similar to this technology but used for a different purpose:

No licensed drugs/therapeutics on the market

(b) List any companies you believe may be interested in this technology:

(b) (4)

(c) If you have a contact at any of these companies, please provide name, email and phone numbers for each, if available:

List the names and organizations of **all people** who participated in conceiving or continued development of the discovery/invention. Examples include those who made intellectual, theoretical, or innovative contribution to the discovery. In the case of software, those individuals who were involved in creating program code, manuals, flowcharts or any related items.

1. Submitting Contributor	(b) (4)	Organization:	(b) (4)
2. Co-Contributor		Organization:	
3. Co-Contributor	John Mascola	Organization:	NIH
4. Co-Contributor	Barney Graham	Organization:	NIH
5.	Jason McLellan		UT Austin

6. Enter additional Co-Contributor's names and organizations as necessary:

Kizzmekia Corbett, NIH; Lingshu Wang, NIH; Julie Ledgerwood, NIH, Olubukola Abiona, NIH, Wei Shi, NIH, Wing-pui Kong, NIH, Yi Zhang, NIH, Nianshuang Wang, UT Austin, Daniel Wrapp, UT Austin

An Additional Contributor Information document (to follow) is to be completed for each contributor listed above. If required, extra forms may be downloaded at: http://www.ott.nih.gov/sites/default/files/documents/docs/eir-additional-contributor.docx

In addition to the above names, identify any individuals who could merit authorship credit of any associated publication:

NOTICE: There may be fewer individuals listed as contributors than named as coauthors. *Please be aware that inventorship is strictly defined in patent law. Accordingly, contributors you list in this section will be named on patent applications resulting from this EIR only if their contributions meet this legal standard.* A co-author may or may not qualify based on the particular facts; if you have any questions, contact your TDC.

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an **obligation to report** and assign inventions to the United States of America as represented by the Government of the United States (the Department of Health and Human Services). Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by HHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your TDC and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

Detailed Contributor Information begins on next page

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 1

Name

♦First		♦Middle		♦Last		Suffix	
Degree		♦Citizenship					
♦HHS ID # (e.g. 999-9999-999)				♦Associated project NIH Z01 Project #			

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title							
♦Office Address							
♦City		♦State		♦Zip		♦Country	
♦Email		Telephone		Fax		Other contact# (optl)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street		♦City		♦State		♦Zip code	
♦ Country		Phone		♦Email			

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 2

Name

♦First		♦Middle		♦Last		Suffix	
Degree		♦Citizenship					
♦HHS ID # (e.g. 999-9999-999)				♦Associated project NIH Z01 Project #			

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title							
♦Office Address							
♦City		♦State		♦Zip		♦Country	
♦Email		Telephone		Fax		Other contact# (optl)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street		♦City		♦State		♦Zip code	
♦ Country		Phone		♦Email			

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	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 3

Name

♦First		♦Middle		♦Last		Suffix	
Degree		♦Citizenship					
♦HHS ID # (e.g. 999-9999-999)				♦Associated project NIH Z01 Project #			

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title							
♦Office Address							
♦City		♦State		♦Zip		♦Country	
♦Email		Telephone		Fax		Other contact# (optl)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street		♦City		♦State		♦Zip code	
♦ Country		Phone		♦Email			

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

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	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

Employee Discovery and Invention Report (EIR)
Contributor Information Sheet

♦ Indicates a required field

Contributor 4

Name

♦First		♦Middle		♦Last		Suffix	
Degree		♦Citizenship					
♦HHS ID # (e.g. 999-9999-999)				♦Associated project NIH Z01 Project #			

♦Describe this individual's contribution to the discovery.

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title							
♦Office Address							
♦City		♦State		♦Zip		♦Country	
♦Email		Telephone		Fax		Other contact# (optl)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain and provide affiliation at time of discovery:

Home Information (will be used for royalty distribution when applicable)

♦Street		♦City		♦State		♦Zip code	
♦Country		Phone		♦Email			

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	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below)*
	Oxford-Cambridge Scholars Program		National Research Council Award		Research Fellowship		NIH Contract Employee – specify employer name *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Signature **Date**

♦ Indicates a required field

Information about this Form

Reporting an invention is required as part of your Government service, and supports the mission of your IC and the NIH in advancing public health. An EIR should be completed for each discovery or invention* that is:

a) An innovation;
b) A new or improved method or process;
c) Believed to have potential commercial value (e.g. a new reagent, unique antibody, vaccine, medical device, or therapeutic compound); **or**
d) Requested from a commercial organization for use or resale.

If you are employed by HHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

IC means a Public Health Service (PHS) Contributor's Institute, Center, or Office (includes NIH, FDA and CDC).

COMPLETION OF THE EIR

1. Complete the form by filling in the shaded fields. For "check boxes" insert "X";
2. Once completed, have each contributor sign their Contributor Information Sheet;
3. Questions regarding the completion of the EIR should be referred to your TDC;
4. Email the completed electronic EIR template and any related documents to your TDC; and
5. After review by your TDC, email a signed copy of the final EIR to your TDC.
6. The TDC will then forward the completed and signed EIR to the Office of Technology Transfer (OTT). If your IC in decides not to file a patent application on your invention you may contact your TDC to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

General questions regarding the form may be directed to your TDC or the NIH Office of Technology Transfer (OTT). It is suggested, particularly if you leave government service and are receiving royalties, that you keep the Office of Financial Management apprised of changes in your official address.

Thank you for your contribution toward improving public health!

Privacy Act Notice: HHS is collecting this information under authority of 45 CFR Part 7 "Employee Inventions". The information will be maintained as a part of the System of Records: 09-25-0168, "Invention, Patent and Licensing Documents." Provision of this information is mandatory and will be used as the initial step toward obtaining patent protection of inventions submitted by HHS employees, granting licenses to HHS inventions, administering and providing royalty payments to HHS inventors, and the intended "routine uses" of the information. Failure to provide complete information may adversely affect the Government's rights to future patent applications and licensing agreements.

***What is the difference between a discovery and an invention?**

Discovery

- Elucidating something that already exists
- Embodied in Nature
- Discovery involves describing something
- Product of Nature
- e.g. A botanist discovers a new plant species on an island

Invention

- An innovation that did not previously exist
- Embodied in human-made artifact
- Always involves creating something
- Produced through human thought
- A botanist invents a new topical antibiotic formulation using the plant oils

Employee Discovery and Invention Report (EIR)
Administration Section
(To be completed by the IC Technology Transfer Office)

♦ Indicates a required field

Lead IC sponsoring this invention:		Division/Lab/ Branch	
Identify any other ICs (if necessary, include Division/Lab/Branch			
Confirm the scientist who will serve as the key point of contact (Scientific Point of Contact or SPC) to receive and respond to patent correspondence. This may be different from the person identified as the Submitting Contributor.			

IC CRADA Determination: (Answer required if the EIR is related to a CRADA)

	The invention was conceived or first actually reduced to practice in the performance of the activities under the CRADA Research Plan during the term of the CRADA. Enter Y or N.

IC is requesting the following action regarding this EIR. *Only 1 of the following option boxes should be checked.*

Do Not File

	Do not file; EIR is being submitted due to a request for material(s) licensing. Requester's contact information is attached
	Do not file; market as a research material
	Do not file a patent application based on other patent and/or policy reasons

Evaluation/Searching

	Request evaluation and recommendation, Outside search/opinion are authorized, if needed
	Request evaluation and recommendation, Outside search/opinion is not authorized

Patent Application Filing

	Emergency Patent Filing - File immediate provisional patent application based on an imminent publication. Authorization for filing is provided. In the "Additional Information or Instructions" box enter the date, location of public release and any other instructions
	File Patent: File a fully enabled provisional patent application
	File Patent/Patent Opinion: File a fully enabled provisional patent application and conduct a patentability opinion/assessment immediately after the provisional patent application has been filed
	Patent Opinion/File Patent: Conduct a patentability opinion/assessment (Completed by contract law firm). If the patentability opinion/assessment is positive, proceed with the filing a fully enabled provisional patent application
	Third Party Filing Patent Lead: Third party has already filed or will be doing the filing. Please provide 3rd Party contact information. Application number(s), filing date(s), and copy(ies) of application(s) should be provided as well as filing receipts or paperwork identifying the inventors as filed
	Other Filing Instructions

Additional Information or Instructions

Is the IC submitting the EIR represented by a Service Center?

If yes, identify IC:

All patent recommendations and patent correspondence will be directed the Lead IC's central email account.

Name of LPM designated for this EIR by IC:

The TDC, IC delegate, or Service Center Representative confirms receiving the EIR and acknowledges a complete EIR packet is being forwarded to OTT.

Name		Date	
Title			

Signature_____

Authorized IC official for expenditure of IC funds for patent related expenses or attach authorization memo.

Name		Date	
Title			

Signature_____

An OCR'd PDF containing the signed EIR containing all documents such as manuscripts, presentations, articles and citations referred to in the EIR, as well as any related IC reviews and authorization documents should be forwarded to OTT at ottfileroom@mail.nih.gov. The email's subject line should include "New EIR for IC, (PI's Last Name), (IC Ref. #, if exists)." Original formatted documents, i.e. MS Word, PowerPoint, should be attached for EIRs with recommendations of "Evaluate", "File Patent", "Patent Opinion" or any combination of these.

Employee Discovery and Invention Report (EIR)
Appendix A - Supplemental Information Sheet for Research Materials

This attachment requests information regarding materials that may be available for potential licensing. The **Set 1** questions elicit general information regarding specific materials required to practice the invention or ancillary materials created during the course of development that may be potentially licensed. The **Set 2** questions SHOULD BE ANSWERED IF THIS EIR is being submitted based on an outside party's request for licensing a material.

Set 1. General material information. *(Not required if Set 2 Questions are completed)*

a) Identify those materials made during the course of research that are specifically required to practice this invention. Please identify each unique material or chemical compound developed that are related to this EIR.

b) Identify those material(s) made during the course of research that may be available for licensing as a research material. Please identify each unique material or chemical compound developed that are related to this EIR.

c) Material citation or other source:

d) Has material been deposited in the ATCC or similar repository? If yes, please provide the repository and catalog reference number.

e) Were any of the materials necessary to use or make the Material acquired from someone outside NIH? If yes and not already listed in Question 5, please provide contact information and a copy of any document that records this transfer.

Set 2. If a prospective licensee is interested in a material answer the following questions.

Provide a summary regarding the approximate difficulty/time/cost/effort for your laboratory to provide the material to an outside for-profit requestor. Is it a limited resource?

Identify and describe the Material Type:

Examples of potential material types:

Antibodies: monoclonal, polyclonal
Cell Lines: uninfected cells, infected cells, hybridomas
DNA/RNA: genetic clones, expression vectors
PCR reagents
Proteins/Peptides
Purified Proteins
Viruses: virus isolates, drug resistant, virus isolates, recombinant vaccinia
Opportunistic Infections: (e.g. Candida, Cryptococcus, cryptosporidium, cytomegalovirus, mycobacterium, mycoplasma, pneumocystis, toxoplasma)
Model Organisms (e.g. strain, species)
Chemical Compounds

Designation: (Laboratory nomenclature)

Source of Material: (i.e. human, mouse, rat)

Reference Citation or other source:

Has the material been deposited in the ATCC or similar repository? If Yes, provide repository & catalog. ref.#s.

How can it be provided: (i.e. 2ml vial of frozen cells, plasmid)

Current quantities available for distribution: (i.e. 10 vials)

Recommended propagation medium & growth characteristics: (i.e. expression level, titer, temp., passages)

Recommended freeze medium:

Sterility: (i.e. negative for bacteria, fungi and mycoplasma)

Morphology: (i.e. epithelial-like, lymphoblast-like, fibroblast-like)

Recommended Storage: (i.e. liquid nitrogen)

Employee Discovery and Invention Report (EIR)
Appendix B - Supplemental Information Sheet for Software

The purpose of this attachment is to provide information regarding evaluation of software for potential licensing. The following questions should be answered as completely as possible.

1. Does this software contain code obtained from a third-party or covered by any Open Source License (e.g., collaborator, under a software agreement, a vendor, purchased etc.)? If Yes, please explain.
2. Has this software been previously copyrighted? If yes, by whom?
3. Did you use outsiders to beta-test code? If yes, was this done under an agreement?
4. How would the lab generally classify the use of this software (e.g., imaging, array analysis, mapping etc.)?
5. From the lab's perspective, what would be the preferred way of distributing this software?
6. What are the operating system requirements to run this software?
7. In which computer language is the software code written?
8. What stage of development is the software? Select one of the following:
   - ☐ Ready to use by anyone
   - ☐ Useable with some effort or assistance
   - ☐ Needs substantial further development
9. Is the lab willing to release the source code?
10. Is the lab willing to prepare a demonstration version of the software to give to prospective licensees?
11. Were the current or prior versions distributed? If yes, explain and supply date of distribution and any distribution agreement (if any).
12. Was a government contractor involved in the writing or development of any of the code? If yes, identify the individual.

Employee Discovery and Invention Report (EIR)
Additional Contributor Information

The Additional Contributor Information document should be used when an EIR has already been completed and there are more than four (4) Contributors identified in the EIR. It is to be completed by any individual whose name was listed as a Contributor, but their Contributor information was not provided in EIR Question 7.

If you are employed by PHS it is presumed that the invention was made as part your official duties as a Government employee. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made.

COMPLETION OF THE CONTRIBUTOR INFORMATION PAGE

1. Review the EIR
   - download the fill able electronic Additional Contributor Information Page WORD template form.
   - complete the Contributor Information Page found on the next page by filling in the shaded fields. For"check boxes" insert "X".
   - questions regarding this EIR should be referred either to NIH/FDA Technology Development Coordinator (TDC)
   - print and sign the form
   - email the completed Contributor Information Page to NIH/FDA Technology Development Coordinator (TDC); and
   - submit the printed and signed EIR to the Agency's TDC.

2. Upon receipt, the TDC will forward your information as part of the completed electronic EIR report and the printed, signed EIR to the Office of Technology Transfer (OTT). If your IC in conjunction with the OTT decides not to file a patent application on your invention you may contact your Technology Development Coordinator (TDC) to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

   Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

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Thank you for your cooperation

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Contributor Information

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Contributor 1

Name

♦First	Julie	♦Middle	E.	♦Last	Ledgerwood	Suffix	
Degree	D.O.	♦Citizenship	(b) (6)				
♦Employee Identification No.:	0011201415			♦Associated project NIH Z01 Project Number(s)		AI005037	

♦**Describe this individual's contribution to the discovery.**

(b) (4)

Employee Discovery and Invention Report (EIR)
Contributor Information

(b) (4)

Current Organization Information:

♦ Organization Name	National Institute of Allergy and Infectious Disease, National Institutes of Health						
Division/Branch/Laboratory	Vaccine Research Center						
♦ Title	Chief Medical Officer and Deputy Director						
♦ Office Address	40 Convent Drive, Bldg 40 Rm 5512						
♦ City	Bethesda	♦ State	MD	♦ Zip code	20892	♦ Country	USA
♦ E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦ Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦ City	Bethesda	♦ State	MD	♦ Zipcode	(b) (6)	♦ Country	USA
Phone	(b) (6)	Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Julie Ledgerwood, D.O. Digitally signed by Julie Ledgerwood, D.O. Reason: I attest to the accuracy and integrity of this document Date: 2020.03.23 13:19:46 -04'00'

Signature **Date**

Employee Discovery and Invention Report (EIR)
Additional Contributor Information

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   - print and sign the form
   - email the completed Contributor Information Page to NIH/FDA Technology Development Coordinator (TDC); and
   - submit the printed and signed EIR to the Agency's TDC.

2. Upon receipt, the TDC will forward your information as part of the completed electronic EIR report and the printed, signed EIR to the Office of Technology Transfer (OTT). If your IC in conjunction with the OTT decides not to file a patent application on your invention you may contact your Technology Development Coordinator (TDC) to request a waiver and, if granted, have an opportunity to obtain the rights to the invention by filing the patent application at your own expense.

   Frequently Asked Questions: http://ottintranet.od.nih.gov/EIR/EIR_FAQS_20110915.htm

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Thank you for your cooperation

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Contributor Information

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Contributor 1

Name

♦First	Kizzmekia	♦Middle	Shanta	♦Last	Corbett	Suffix	
Degree	PhD	♦Citizenship	(b) (6)				
♦Employee Identification No..	0012686509	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

(b) (4)

Current Organization Information:

♦Organization Name							
Division/Branch/Laboratory							
♦Title	Research Fellow						
♦Office Address	40 Convent Drive, Room 2608, Bethesda MD 20902						
♦City	Bethesda	♦State	MD	♦Zip code	20902	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	(b) (6)

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

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	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award	X	Research or Clinical Fellowship		NIH Contract Employee – specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

Employee Discovery and Invention Report (EIR)
Contributor Information

(b) (6)

March 23, 2020

Signature Date

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	John	♦Middle	R	♦Last	Mascola	Suffix	
Degree	MD	♦Citizenship	(b) (6)				
♦Employee Identification No.:	001-1058-566	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

(b) (4)

Current Organization Information:

♦Organization Name	Vaccine Research Center, NIAID						
Division/Branch/Laboratory	Virology Laboratory						
♦Title	Director, Vaccine Research Center						
♦Office Address	40 Convent Drive, Bldg 40, Rm 4504						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EI

(b) (6)

Signature **March 22, 2020**

Date

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Yi	♦Middle		♦Last	Zhang	Suffix	
Degree	B.S	♦Citizenship	(b) (6)				
♦Employee Identification No.:	2000750029	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery**.

(b) (4)

Current Organization Information:

♦Organization Name	NIAID, NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Biologist						
♦Office Address	Room 4606B, Building40, 40 Convent Drive						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	US
Phone		Email					

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section — Contract employer: Kelly Government

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

Signature

3/22/2020

Date

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Lingshu	♦Middle		♦Last	Wang	Suffix	
Degree	Ph.D.	♦Citizenship	(b) (6)				
♦Employee Identification No.	0013473896	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery**.

Contributed the strategies for more effectively isolating mAbs; Developed neutralization assay to test the function of these mAbs; Participated in onging discussions regarding how to analyze data and choose lead mAbs

Current Organization Information:

♦Organization Name	NIAID, NIH						
Division/Branch/Laboratory	Vaccine Research Center						
♦Title	Staff Scientist						
♦Office Address	Room 4608B, Building 40, 40 Convent Drive						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	US
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX		Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	US
Phone		Email					

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee - specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

(b) (6)

3/24/20

Date

Employee Discovery and Invention Report (EIR)
Contributor Information

The following acknowledgement pertains to Government employees and those treated as employees. Under 45 CFR Part 7 "Employee Inventions", all employees of the Public Health Service have an obligation to report and assign inventions to the Government of the United States, as represented by the Department of Health and Human Services. Specifically, the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) made with a contribution by the Government of facilities, equipment, materials, funds or information or of time or services of other Government employees on official duty; or (iii) which bear a direct relationship or are made in consequence of the official duties of the inventor.

If you are employed by PHS to conduct or perform research, it is presumed that the invention was made under these circumstances. If this is not the case, you should still complete the EIR, but you must contact your Technology Development Coordinator (TDC) and provide the details pertaining to this particular discovery or invention so that a determination of rights can be made..

Contributor 1

Name

♦First	Wing Pui	♦Middle		♦Last	Kong	Suffix	
Degree	Ph.D.	♦Citizenship	(b) (6)				
♦Employee Identification No..	0010151956	♦Associated project NIH Z01 Project Number(s)					

♦**Describe this individual's contribution to the discovery.**

Directed and developed a new COVID-19 neutralization assay. Directed and performed mAb characterization: measure and analyze neutralization potency of mAbs and epitope binding characteristics and collaborate to choose lead candidates

Current Organization Information:

♦Organization Name	Vaccine Research Center						
Division/Branch/Laboratory	NIAID/NIH						
♦Title	Staff Scientist						
♦Office Address	40 Convent Drive						
♦City	Bethesda	♦State	MD	♦Zip code	20892	♦Country	USA
♦E-mail	(b) (6)	Telephone	(b) (6)	FAX	(b) (6)	Other contact number. (optional)	

♦Has your organizational affiliation changed during the development of this discovery? Yes/No, if yes, explain

No

Home Information

♦City	(b) (6)	♦State	MD	♦Zipcode	(b) (6)	♦Country	USA
Phone		Email	(b) (6)				

Please identify with a "X" if this individual falls under one or more of the following training or fellowship appointments or institutional partnerships.

	CRADA Personnel		Howard Hughes Fellow		ORISE Fellow		NIH-ORAU
	Clinical Fellow		Gates Foundation		NRSA Fellowship		Visiting Fellowship
	Fogarty Scholar		IRTA Fellowship Program		Postdoctoral Fellow		Other (specify below *
	Oxford-Cambridge Scholars Prog		National Research Council Award		Research or Clinical Fellowship		NIH Contract Employee – specify employer name below *
	CNRM Personnel (HJF)		Society Fellows specify below		Graduate Partnership Program		

* Note Section

Contributor: I have read and understand the information submitted in the EIR.

_Kong Wing Pui_____ ____3-24-2020_____

ORIGINAL

APR 30 2004

For TTO Use

CDC Ref. #
I-005-04
U.S.P.A.#

U.S. Filing *(date)*

CDC Employee Invention Report

Use plain paper if more space is needed.

Part I: To Be Completed by the Inventor

First Inventor's Name: Olen M. Kew

Phone No. 404-639-3940

1. Give a short descriptive title of your discovery or invention.

   Modulation of poliovirus replicative fitness by deoptimization of synonymous codons

2. Please provide (in non-scientific terms if possible) a one paragraph description of the essence of your discovery or invention and identify the public health need it fills.

   See attached

3. Who contributed to the invention or discovery? Please identify all colleagues who *could* merit co-authorship credit for the associated publication, whether or not you believe them to be "co-inventors."

   Cara Carthel Burns, Ph.D., Jing Shaw, M.D., Raymond Campagnoli, M.S., Jacqueline Quay, M.S., Jaume Jorba, M.S., Annalet Vincent, B.S.

4. Is anyone outside of the Public Health Service aware of your invention or discovery? If so, please identify them and describe the dates and circumstances.

   No, apart from a few overseas visitors who came to the laboratory for training in poliovirus molecular diagnostics.

5. Are you aware of any PHS patent applications that are related to your invention or discovery?

   No

6. Please list the most pertinent previous articles, presentations or other public disclosures, made by you or by other researchers, that are related to your invention or discovery. Also, attach four sets of copies, ***please!***

   See attached list

7. Please indicate any future dates on which you will publish articles or make *any* presentations related to your invention or discovery.
The first manuscript (attached) describing our invention is in preparation for submission to the J. Virology in mid-2004.

8. In one paragraph, please speculate (and be creative!) about possible commercial uses of your invention or discovery.
See attached

9. a. Is the subject matter of your invention related to a PHS CRADA (Cooperative Research and Development Agreement) involving your laboratory or CIO?
☑ No
☐ Yes. If yes, please identify the collaborator: _____

b. Is the subject matter based on research materials that you obtained from some other laboratory?
☑ No
☐ Yes. If yes, please attach any material transfer agreements (MTA) under which you received the material.

10. What companies or academic research groups are conducting similar research (if you know)? Can you identify any companies that may be good licensing prospects?
Codon optimization is a standard procedure in biotechnology to increase yields of proteins expressed in heterologous systems; codon deoptimization is normally avoided.
Licensing: Donate to WHO for polio vaccines; license to vaccine manufacturers for other applications

11. What further research would be necessary for commercialization of your invention? Generally, what are your future research plans for the invention and/or for research in areas related to the invention?
* Explore molecular parameters controlling fitness
* Investigate attenuation of codon-deoptimized MEF-1 constructs in animal (mouse) models; the reference Sabin 2 strain is highly attenuated for mice, so the mouse-virulent MEF-1 strain will be used to test effects of codon-deoptimization
* Further investigate genetic stability of possible attenuated phenotype
* Develop Salk IPV strain constructs fully deoptimized for specific unpreferred codons; attempt to restore efficient replication in production VERO cells overexpressing corresponding minor tRNAs; determine infectivity of constructs in experimental animals; explore suitability of system for IPV production to achieve higher levels of biological containment thereby reducing the risk that virulent IPV seed stocks could spread to the community from an IPV production facility
* Investigate possible applications of codon-deoptimization for other biological systems, especially in the area of attenuated vaccine development; seek collaborations with leading laboratories within and outside of CDC specializing in other important infecious agents

12. Human Subject Certification: Does this invention rely upon data involving human subjects as defined in and regulated under 45 CFR Part 46?
☑ No ☐ Yes → If "yes," please provide the Institutional Review Board (IRB) protocol approval number and date: _____ ***or*** explain fully below:

13. First Inventor Information: *(Provide this information for each inventor who contributed to the essence of the invention. If more than one, use Page 4, "Information on Additional Inventors."*

Name: Olen M. Kew | Degree: Ph.D. | Social Security No. *(optional)*

Position Title: Team Leader, Molecular Virology | Office address: Mailstop G-10 (17-6048)

Office Phone No.: 404-639-3940 | FAX No.: 404-639-4011 | Citizenship: (b) (6)

Home address: (b) (6)

Affiliation

☑ CIO *(specify CIO and applicable box below)* NCID/DVRD/REVB/Molecular Virology Laboratory

☑ GS ☐ CO ☐ Visiting Scientist ☐ Special Volunteer

☐ GM ☐ Visiting Fellow ☐ Howard Hughes Fellow ☐ Other *(specify):*

☐ SES ☐ Visiting Associate ☐ Guest Researcher

☐ Non CIO Affiliation *(specify):*

If more than one inventor, what specific contribution did you make to this work?

Originated general concept of codon deoptimization project, and suggested general experimental approach.

14. Inventors' Signatures

► This report is submitted pursuant to Executive Order 10096 and 10930 and/or Department Regulations. PHS employees have an obligation to report inventions they make while employed by PHS to TTO. Under E.O. 10096 and 367 CFR 501 the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) with Government facilities, equipment, materials, funds or information; or (iii) which bear a direct relationship or is made in consequence of the official duties of the inventor. If you are employed by PHS to conduct or perform research it is presumed that the invention was made under the foregoing circumstances. If this is not the case you must contact your Technology Development Coordinator (TDC) and provide the TDC with the details pertaining to this particular discovery or invention so that a determination of rights can be made.

Inventors' Signatures	Dates	Witnessed Signatures	Dates
(b) (6)	4.28.04	(b) (6)	4/28/04
	4.28.04		
	4.28.04		
	4-28-04		
	5/5/04		

Part II: To be completed by the Technology Development Coordinator.

15. Institute(s) or Agency(s) sponsoring this invention

16. Patent prosecution fees are to be charged to

CAN:

CIO:

Authorizing Official *(Typed)* | Signature | Date

► ***Send signed original and 3 copies of this form to TTO.***

Information on Additional Inventors *(copy this page as needed)*

Name: Cara Carthel Burns | Degree: Ph.D. | Social Security No. *(optional)*:

Position Title: Microbiologist | Office address: Mailstop G-10 (17-6054)

Office Phone No.: 404-639-5499 | FAX No.: 404-639-4011 | Citizenship: (b) (6)

Home address: (b) (6)

Affiliation

☑ CIO *(specify CIO and applicable box below)*: NCID/DVRD/REVB/Molecular Virology Laboratory

- ☑ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☐ Other *(specify)*:

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

Guided team for codon-deoptimization project; designed most of the specific complex series of experiments (and directly performed many) leading to production of codon-deoptimized Sabin 2 and MEF-1 strains.

Name: Jing Shaw | Degree: M.D. | Social Security No. *(optional)*:

Position Title: Associate Service Fellow | Office address: Mailstop G-10 (17-6054)

Office Phone No.: 404-639-3592 | FAX No.: 404-639-4011 | (b) (6)

Home address: (b) (6)

Affiliation

☑ CIO *(specify CIO and applicable box below)*: NCID/DVRD/REVB/Molecular Virology Laboratory

- ☐ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☑ Other *(specify)*: Assoc. Service Fellow

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

Conducted many of the critical experiments leading to the development and characterization of the codon-deoptimized Sabin 2 and MEF-1 constructs; designed key primers for MEF-1 constructs.

Name: See next page | Degree: | Social Security No. *(optional)*:

Position Title: | Office address:

Office Phone No.: | FAX No.: | Citizenship: ☐ U.S. ☐ Other:

Home address:

Affiliation

☐ CIO *(specify CIO and applicable box below)*

- ☐ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☐ Other *(specify)*:

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

► ***Send signed original and 3 copies of this form to TTO.***

Information on Additional Inventors *(copy this page as needed)*

Name: Raymond Campagnoli | Degree: M.S. | Social Security No. *(optional)*:

Position Title: Microbiologist | Office address: Mailstop G-10 (17-6023)

Office Phone No.: 404-639-0806 | FAX No.: 404-639-4011 | Citizenship: (b) (6)

Home address: (b) (6)

Affiliation

☐ CIO *(specify CIO and applicable box below)* NCID/DVRD/REVB/Molecular Virology Laboratory

- ☑ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☐ Other *(specify)*:

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

Participated in the experimental characterization of Sabin 2 constructs. Constructed and characterized infectious MEF-1 clone, and prepared and characterized codon-deoptimized MEF-1 constructs.

Name: Jacqueline Quay | Degree: M.S., J.D. | Social Security No. *(optional)*:

Position Title: Microbiologist (at time) | Office address: K-79 (Koger WMS 3832)

Office Phone No.: 770-488-8608 | FAX No.: 770-488-8615 | Citizenship: (b) (6)

Home address: (b) (6)

Affiliation

☑ CIO *(specify CIO and applicable box below)* NCID/DVRD/REVB/Molecular Virology Section (at time)

- ☑ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☐ Other *(specify)*:

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

Conducted many of the critical initial experiments leading to the development of the Sabin 2 infectious clone and the codon-deoptimized Sabin 2 construct.

Name | Degree | Social Security No. *(optional)*:

Position Title | Office address

Office Phone No. | FAX No. | Citizenship ☐ U.S. ☐ Other:

Home address

Affiliation

☐ CIO *(specify CIO and applicable box below)*

- ☐ GS
- ☐ GM
- ☐ SES
- ☐ CO
- ☐ Visiting Fellow
- ☐ Visiting Associate
- ☐ Visiting Scientist
- ☐ Howard Hughes Fellow
- ☐ Guest Researcher
- ☐ Special Volunteer
- ☐ Other *(specify)*:

☐ Non-CIO Affiliation *(specify)*:

What specific personal contribution did she/he make to this work?

2. Brief description of invention

The attenuated Sabin oral polio vaccine (OPV) strains are genetically unstable, principally because only 2 – 5 base substitutions confer the attenuated phenotype (7). This instability is the underlying cause of vaccine-associated paralytic poliomyelitis in immunologically normal (8) and immunodeficient people (4, 6), and of outbreaks associated with circulating vaccine-derived polioviruses (3, 5). We sought to increase the genetic stability of the OPV strains by distributing additional new attenuating mutations over many sites within the genome, such that the observed phenotype reflects the cumulative effects of many substitutions, each incrementally reducing replicative fitness (i.e., attenuation). To achieve this, we engineered changes in the codon composition of the capsid region (which encodes for all poliovirus antigens) without changing the encoded amino acid sequence, forcing the virus to use rare synonymous codons and low-abundance tRNAs for translation (i.e., protein synthesis). In both the Sabin type 2 OPV strain and the Salk MEF-1 type 2 inactivated polio vaccine (IPV) strain, the extent of fitness decrease was linearly proportional, over a 100-fold range, to the length of the interval containing replacement codons. Full reversion of the attenuated phenotype may require back-mutation at many sites, thereby conferring enhanced genetic stability to this important virus trait. Because all replacement codons were synonymous, the encoded proteins (and thus, antigenic properties) were identical to those of the prototype strains.

We are currently investigating the underlying molecular mechanisms of the observed fitness reductions, which most likely involve ribosome stalling during translation, as well as other effects currently not well understood. Apart from the relevance of these findings to basic science, codon deoptimization may have important applications in the development of more stable attenuated vaccines and safer production conditions for inactivated vaccines. The principles established here with polioviruses potentially have broad applications, cutting across, and quite possibly beyond, the world of microbial pathogens.

The impetus for this work was the desirability of safer OPV and IPV strains for the global polio eradication endgame (2). It is probably now unrealistic to expect that new OPV strains could be tested and introduced at this late stage of global polio eradication (1, 2). However, IPV is likely to be used for the foreseeable future, and codon-deoptimized seed stocks, incapable of efficiently infecting humans but capable of infecting appropriately engineered cells, may have prospects for use. They might substantially reduce the risks of inadvertent infection of workers producing IPV from high titers of the virulent Salk IPV strains, thereby strengthening poliovirus containment and further reducing the risk that poliovirus could be inadvertently released into the community. This approach, if found to provide additional biological containment within IPV production facilities, may also provide similar protections in other facilities producing inactivated vaccines from pathogenic seed stocks, such as for foot-and-mouth disease virus (FMDV; like poliovirus, a picornavirus) and influenza virus.

Other potential applications would be the genetic stabilization of other RNA virus vaccines, and the development of new, stable attenuated vaccines by precise modulation of replicative fitness of bacterial or protozoan pathogens through the deoptimization of synonymous codon usage of critical single-copy genes.

1. **Aylward, R. B., and S. L. Cochi.** 2004. Framework for evaluating the risks of paralytic poliomyelitis after global interruption of wild poliovirus transmission. Bull. WHO **82:**40-46.
2. **Dowdle, W. R., E. de Gourville, O. M. Kew, M. A. Pallansch, and D. J. Wood.** 2003. Polio eradication: the OPV paradox. Rev. Med. Virol. **13:**277-291.
3. **Kew, O. M., V. Morris-Glasgow, M. Landaverde, C. Burns, J. Shaw, Z. Garib, J. André, E. Blackman, C. J. Freeman, J. Jorba, R. Sutter, G. Tambini, L. Venczel, C. Pedreira, F. Laender, H. Shimizu, T. Yoneyama, T. Miyamura, H. van der Avoort, M. S. Oberste, D. Kilpatrick, S. Cochi, M. Pallansch, and C. de Quadros.** 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science **296:**356-359.
4. **Kew, O. M., R. W. Sutter, B. Nottay, M. McDonough, D. R. Prevots, L. Quick, and M. Pallansch.** 1998. Prolonged replication of a type 1 vaccine-derived poliovirus in an immunodeficient patient. J. Clin. Microbiol. **36:**2893-2899.
5. **Kew, O. M., P. F. Wright, V. I. Agol, F. Delpeyroux, H. Shimizu, N. Nathanson, and M. A. Pallansch.** 2004. Circulating vaccine-derived polioviruses: current state of knowledge. Bull. WHO **82:**16-23.
6. **Khetsuriani, N., D. R. Prevots, L. Quick, M. E. Elder, M. Pallansch, O. Kew, and R. W. Sutter.** 2003. Persistence of vaccine-derived polioviruses among immunodeficient persons with vaccine-associated paralytic poliomyelitis. J. Infect. Dis. **188:**1845-1852.
7. **Ren, R., E. G. Moss, and V. R. Racaniello.** 1991. Identification of two determinants that attenuate vaccine-related type 2 poliovirus. J. Virol. **65:**1377-1382.
8. **Strebel, P. M., R. W. Sutter, S. L. Cochi, R. J. Biellik, E. W. Brink, O. M. Kew, M. A. Pallansch, W. A. Orenstein, and A. R. Hinman.** 1992. Epidemiology of poliomyelitis in the United States one decade after the last reported case of indigenous wild virus-associated disease. Clin. Infect. Dis. **14:**568-579.

6. Previous pertinent articles

The original article on codon-deoptimization was:

Hoekema, A., R. A. Kastelein, M. Vasser, and H. A. de Boer. 1987. Codon replacement in the *PGK1* gene of *Saccharomyces cerevisiae*: experimental approach to study the role of biased codon usage in gene expression. Mol. Cell Biol. **7:**2914-2924.

Other pertinent codon-deoptimization articles that appeared after the start of our project were:

Carlini, D. B., and W. Stephan. 2003. In vivo introduction of unpreferred synonymous codons into the Drosophila Adh gene results in reduced levels of ADH protein. Genetics **163:**239-243.

Zhou, J., W. J. Liu, S. W. Peng, X. Y. Sun, and I. Frazer. 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. J. Virol. **73:**4972-4982.

Pioneering articles on codon-optimization were:

Bennetzen, J., and B. Hall. 1982. Codon selection in yeast. J. Biol. Chem. **257:**3026-3031.

Robinson, M., R. Lilley, S. Little, J. S. Emtage, G. Yarronton, P. Stephens, A. Millican, M. Eaton, and G. Humphreys. 1984. Codon usage can affect efficiency of translation of genes in *Escherichia coli*. Nucleic Acids Res. **12:**6663-6671.

8. Potential applications

- Development of new generation live, attenuated OPV strains with enhanced genetic stabilities.
- Development of codon-deoptimized seed strains for IPV and other inactivated vaccines (e.g., FMDV or influenza) production suitable for large-scale production in engineered host cells for IPV production, but capable of only inefficient replication in humans, thus creating a biological barrier to inadvertent infection of workers in a vaccine production facility.
- Opening a new avenue to develop new live, attenuated vaccines (e.g., for newly discovered agents such as the SARS coronavirus) or improve the genetic stabilities of existing vaccines for RNA and DNA viruses, and bacterial and protozoan pathogens.

Modulation of Poliovirus Replicative Fitness in HeLa Cells by Deoptimization of Codon Usage in the Capsid Region

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Running title: Codon deoptimization in poliovirus

Abstract

We replaced the original capsid region codons of the Sabin type 2 oral poliovaccine strain with synonymous codons less frequently used in poliovirus genomes. An unpreferred synonymous codon was used nearly exclusively to code for each of nine amino acids. Codon changes were introduced into four contiguous intervals spanning 97% of the capsid region. Replicative fitness in HeLa cells, measured by plaque size and virus yields in single-step growth experiments, was inversely proportional to the number of codon-replacement nucleotide substitutions. Virus yields varied over a ~65-fold range in response to the extent of codon deoptimization. In the capsid region of the most highly modified virus construct, the effective number of codons used (N_c) fell from 56.2 to 29.8, the number of CG dinucleotides rose from 97 to 302, and the G + C content increased from 48.4% to 56.4%. Replicative fitness of both modified and unmodified viruses increased with passage in HeLa cells. After 25 serial passages (~50 replication cycles), the relative fitness of the modified viruses remained well below that of the unmodified virus and most codon modifications were preserved. The increased replicative fitness of high-passage modified virus was associated with the elimination of several CG dinucleotides.

INTRODUCTION

Codon usage bias, the use of synonymous codons at unequal frequencies, is ubiquitous among genetic systems (19, 20). The strength and direction of codon usage bias is related to genomic G + C content and the relative abundance of different isoaccepting tRNAs [reviewed in (1, 13, 38)]. Codon usage can affect the efficiency of gene expression. In *Escherichia coli* (19, 60), *Saccharomyces cerevisiae* (4, 20), *Caenorhabditis elegans* (13), *Drosophila melanogaster* (36), and *Arabidopsis thaliana* (8) the most highly expressed genes use codons matched to the most abundant tRNAs (2). By contrast, in humans and other vertebrates, codon usage bias is much more strongly correlated with the G + C content of the isochore where the gene is located (37, 56) than with the breadth or level of gene expression (13) or the number of tRNA genes (21, 23). Despite the weak correlation between codon usage and the levels of gene expression in mammalian cells (13, 56), mismatch between codon usage and tRNA abundance can sharply reduce the levels of gene expression. For example, translation of mRNA encoding the L1 and L2 capsid proteins of papillomavirus is restricted by the prevalence of unpreferred codons in the message (64). The translational limitation could be overcome in vivo by changing the codon composition of the L1 and L2 genes and in vitro by addition of charged tRNAs (64).

Optimization of codon composition is frequently required for efficient expression of genes in heterologous host systems (3, 24, 51, 61). For example, the expression human immunodeficiency type 1 gp120 in mammalian cells (3) and *Plasmodium falciparum* surface antigens in *Pichia pastoris* (61) are enhanced by replacing the natural codons with preferred codons of the most highly expressed genes of the expression system. Conversely, engineered codon deoptimization can dramatically decrease the efficiency of gene expression in *E. coli* (46), *S. cerevisiae* (17), *D. melanogaster* (7), and mammalian cells (64).

Codon usage bias in human RNA viruses generally appears to be low, and differences in codon usage are most strongly correlated with genomic G + C content (22), which range from ~35% in rotavirus (14) to ~70% in rubella virus (11). Codon usage in vertebrate genomic DNA and most eukaryotic RNA viruses is also shaped by the suppression of CG dinucleotides (25). Polioviruses and the closely related species C human enteroviruses have moderate (44% to 47%) G + C contents in their RNA genomes (5), apparently low codon usage bias (22), and low abundance of CG dinucleotides (25, 55).

We have studied the effects of altered codon composition on the replicative fitness of poliovirus type 2. Polioviruses are small (28 nm diameter), non-enveloped viruses whose single-stranded genome is enclosed in a capsid of 60 identical subunits arranged in icosahedral symmetry. Their positive-stranded genomes (~7500 nt) can serve directly as a messenger RNA, which is translated as a large (~250 kD) polyprotein from a single open reading frame (ORF). The polyprotein is co-translationally processed in a proteolytic cascade catalyzed by virus-encoded proteases, producing at least 10 distinct final cleavage products. Polioviruses grow rapidly in a wide variety of cultured human and simian cells, yielding 10^3 to 10^4 infectious particles per infected cell in ~ 8 hours. As with other RNA viruses, the poliovirus replicase lacks proofreading activity and consequently has a very high rate of base misincorporation [~10^{-4} base substitution per base pair per replication (10, 12)]. Because poliovirus causes acute infection of lymphoid tissue of the small intestine and oropharynx, and is normally spread by person-to-person transmission (53), poliovirus replication under natural conditions is probably nearly continuous. The poliovirus genome evolves at the exceptionally high rate of ~10^{-2} nucleotide substitutions per site per year during replication in humans (31, 62). Most of the substitutions are transitions at synonymous third codon-positions (31, 62), and codon usage among

polioviruses (27, 55) and the closely related species C enteroviruses (5) is conserved. Polioviruses exist as three stable serotypes, and for each serotype strains with reduced replicative fitness (the "attenuated" Sabin oral poliovirus vaccine [OPV] strains) have been used throughout the world as live virus vaccines (53).

In this study, we replaced the natural codons of the Sabin type 2 (Sabin 2) OPV strain (49, 55) with synonymous unpreferred codons in sequences encoding the capsid proteins. Virus plaque size and yield in cell culture decreased in proportion to the number of unpreferred codons incorporated into the capsid sequences. The altered codon composition was largely conserved during 25 serial passages in HeLa cells. Fitness for replication in HeLa cells of both the unmodified Sabin 2 and modified constructs increased with higher passage; however, the relative fitness of the modified constructs remained lower than that of the unmodified construct. These observations may be important to the development of improved attenuated RNA virus vaccines with well-defined levels of replicative fitness and enhanced genetic stabilities.

MATERIALS AND METHODS

Virus and cells. The Sabin Original + 2 (49) master seed of the Sabin type 2 oral poliovaccine strain (P712 ch 2ab) was kindly provided by R. Mauler of Behringwerke AG (Marburg, Germany). Virus was grown at 35°C in suspension cultures (48) of S3 HeLa cells (human cervical carcinoma cells; ATCC CCL- 2.2) or in monolayer cultures of HeLa (ATCC CCL-2), and RD (human rhabdomyosarcoma cells; ATCC CCL-136) cells.

Preparation of infectious Sabin 2 clones. Poliovirus RNA was extracted from 250 μl of cell culture lysate (from ~75,000 infected cells) by using TRIZOL LS reagent (Life Technologies, Rockville, Md.) and further purified on CENTRI-SEP columns (Princeton Separations, Adelphia, N.J.). Full-length cDNA was reversed transcribed (42°C for 2h) from ~1 μg of viral RNA in a 20 μl reaction containing 500 μM dNTP (Roche Applied Science, Indianapolis, Ind.), 200 U Superscript II Reverse Transcriptase (Life Technologies), 40 U RNase-inhibitor (Roche), 10 mM dithiothreitol, and 500 ng primer S2-7439A-B [CCTAAGC$(T)_{30}$CCCCGAATTAAAGAAAAATT TACCCCTACA] (9) in Superscript II buffer. After reverse transcription, 2 U RNase H (Roche) was added and incubated at 37°C for 40 min. Long PCR amplification of viral cDNA was performed using TaqPlus Precision (Stratagene, La Jolla, Calif.) and AmpliWax PCR Gem 100 beads (Applied Biosystems, Foster City, Calif.) for "hot start" PCR in thin-walled tubes. The bottom mix (50 μl) contained 200 μM each dNTP (Roche) and 250 ng each of primers S2-7439A-B and S2-1S-C (GTAGTCGACTAATACGACTCACTATAGGTTAAAACAGCTCTGGGGTTG) in TaqPlus Precision buffer. A wax bead was added to each tube, and samples were heated at 75°C for 4 min and cooled to room temperature. The top mix (50 μl) contained 2 μl of the cDNA and 10 U

TaqPlus Precision in TaqPlus Precision buffer. The samples were incubated in a thermal cycler at 94°C for 1 min and then amplified by 30 PCR cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 8 min), followed by a final 94°C for 1 min and final extension of 72°C for 20 min.

PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and sequentially digested for 2 h at 37°C with restriction enzymes Sal I and Hind III prior to gel purification. PCR products were ligated to pUC19 plasmids following standard methods (50) and ligated plasmids were transformed into XL-10 Gold supercompetent *E. coli* cells (Stratagene) according to the manufacturer's instructions. Colonies were screened for recombinant plasmids on X-gal indicator plates (50) and 6 white colonies were transferred to 1.5 ml Luria-Bertani broth containing 50 μg/ml ampicillin (LB/amp) (Roche). Plasmids were purified using QIAprep Spin Miniprep columns and sequences of the inserts were determined by cycle sequencing using an automated DNA sequencer (Applied Biosystems, Foster City, Calif.) (31). The full-length viral insert was sequenced in both orientations using overlapping sense and antisense primers spaced ~500 nt apart. Selected clones were grown in 50 ml LB/amp, and recombinant plasmids were purified using the QIAfilter Plasmid Maxi kit.

Virus Preparation. Plasmids were linearized with Hind III and gel-purified prior to RNA transcription from 1 μg of plasmid DNA using the Megascript T7 In Vitro Transcription kit (Ambion, Austin, Tex.). RNA yields were estimated using DNA Dipsticks (Invitrogen, Carlsbad, Calif.) and RNA chain length was analyzed by electrophoresis on 1% formaldehyde gels prior to transfection. RD cells were transfected with transcripts of viral RNA by using Tfx-20 (Promega, Madison, Wis.). Briefly, semi-confluent RD cells in 12-well cell culture plates were inoculated with 500 μl MEM (MEM incomplete) (Life Technologies) containing 0.1 μg viral RNA transcript and 0.45 μl Tfx-20 Reagent. Plates were incubated for 1 h at 35°C prior to

addition of 1.5 ml MEM complete [MEM incomplete supplemented with 100 U penicillin and 100 μg streptomycin, 2 mM L-glutamine, 0.075% $NaHCO_3$, 10 μM HEPES (pH 7.5)] (Life Technologies) containing 3% fetal calf serum (FCS; HyClone, Logan, Ut.). Negative controls were performed using RNA transcribed from pBluescriptII SK+ (Stratagene) containing a viral insert truncated at base 7200 by digestion with BamHI and transcribed in a reverse orientation from a T3 promoter. Complete CPE was observed after incubation at 35°C for 18–20 h at which point 400 μl from the transfected wells were transferred to a confluent RD cell monolayer in 75 cm^2 flasks containing MEM complete. Complete CPE was observed after 24 h in the second passage, and virus was liberated from the infected cells by three freeze-thaw cycles and clarification by centrifugation for 15 min at 15,000 × g. Control wells were passaged once and monitored for 72 h post-transfection. The sequences of all virus stocks were verified by RT-PCR amplification of two large overlapping fragments and subsequent sequence analysis of the PCR product.

Site-Directed Mutagenesis. Single-base substitutions were introduced by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following to the manufacturers' protocols. Briefly, two complementary primers containing the desired mutation were designed for PCR amplification of the plasmid containing the Sabin 2 insert. Amplification was performed using Pfu Turbo DNA polymerase on 5 ng of template DNA for 15 cycles at 95°C for 30 s, 50°C for 1 min, and 68°C for 23 min. PCR products were digested for 1 h at 37°C with 10 U of Dpn I prior to transformation in XL-1 Blue Supercompetent cells. Colonies were grown and screened by sequencing as described above.

Assembly PCR. Multiple base substitutions were introduced by assembly PCR (52). Primers were designed to span the region of interest with complementary 40-mers overlapping

by 10 nt on each end. A first round of assembly (30 PCR cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 45 s) was performed with a 20 μl reaction mixture containing Taq Plus Precision buffer, 10 U Taq Plus Precision, 5 pmoles of each primer, and 200 μM dNTP. A second round of assembly (25 PCR cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min) was performed using the outermost sense and antisense primers in a 100 μl reaction mixture in Taq Plus Precision buffer containing 2 μl of product from the first assembly round, 10 U Taq Plus Precision, 200 ng of each primer, and 400 μM dNTP. PCR products were column purified prior to digestion, ligation, and transformation into XL-10 gold supercompetent cells. Clones were grown and screened by sequencing of insert as described.

Plaque Assay. Plaque assays were performed by a modification of previously described methods (62). Briefly, confluent HeLa cell monolayers in 100 cm^2 cell culture dishes were washed, inoculated with virus in MEM incomplete, and incubated at room temperature for 30 min prior to the addition of 0.45% SeaKem LE Agarose (BioWhittaker Molecular, Rockland, Me.) in MEM complete containing 2% FCS. Plates were incubated for 60 h at 35°C, fixed with 0.4% formaldehyde and stained with 0.1% gentian violet. Plaque size was quantified by scanning plates on a FOTO/Analyst Archiver system (Fotodyne, Hartland, Wis.) and subsequent image analysis using Scion Image for Windows (Scion Corp., Frederick, Md.).

Single-step growth curves. S3 HeLa suspension cells (1×10^7) were infected at a multiplicity of infection (moi) of 5 PFU/cell with stirring for 30 min at 35°C. After 30 min, cells were sedimented by low-speed centrifugation and resuspended in 2.5 ml warm complete media SMEM containing glutamine, 5% FCS, penicillin-streptomycin, and 25mM HEPES (pH 7.5). Incubation continued at 35°C in a water bath with orbital shaking at 300 rpm. Samples were

withdrawn at 2-hour intervals from 0 to 14 hours postinfection, and titered by plaque assay at 35°C.

Construction of recombinant clones. Our Sabin 2 infectious clone, S2R9 (*ABCD* construct), differed from the published sequence of a reference Sabin 2 strain (45) at 4 synonymous third-codon positions: G_{2616} (in VP1 region; A replaced to introduce an EagI site) T_{3303} (in VP1 region; A replaced to introduce a XhoI site), T_{4290} (in 2C region), A_{5640} (in $3C^{pro}$ region). This modified construct was used as our reference Sabin 2 strain. Recombinant clones having different combinations of blocks of replacement codons were constructed using standard methods (28).

RNA secondary structure analysis. Prediction of the secondary structure of the original and codon-deoptimized RNA templates was performed using the MFOLD program (34).

In vitro translation. RNA transcribed from recombinant plasmids was translated in vitro in rabbit reticulocytes lysates (Promega, Madison, Wis.) supplemented with HeLa cell extract.

Serial passage of recombinant virus in HeLa cells. Poliovirus constructs S2R9, S2R19, and S2R23 were serially passaged in HeLa cell monolayers in T75 flasks at 35°C for 36 h, at an input moi ranging from 0.1 PFU/cell to 0.4 PFU/cell.

RESULTS

Codon usage in poliovirus. Mononucleotide and dinucleotides frequencies, and codon usage were analyzed in the original reports of poliovirus genomic sequences (27, 43, 47, 55). The mono-, di-, and trinucleotide frequency patterns are similar for the three Sabin strains (55) and appear to be generally conserved across poliovirus genotypes (18, 26, 29, 30, 33, 62) and human enterovirus species C serotypes (5). As with other enteroviruses, the component bases in the Sabin 2 ORF are present in approximately equal proportions [24.0% U, 22.9% C, 29.9% A, and 23.1% G (45, 55)], thus permitting a low bias in codon usage (38). Indeed, all codons are used in poliovirus ORFs (55), and the overall degree of codon usage bias is low (22). One measure of codon usage bias is the number of effective codons (N_C), which can vary from 20 (only one codon used for each amino acid) to 61 (all codons used randomly) (59). The N_C values for Sabin 2 are 56.0 for the capsid region and 54.6 for the complete ORF. As with the genomes of vertebrates and nearly all RNA viruses (25), the dinucleotide CG is suppressed in the Sabin 2 genome (55), and the observed pattern of codon usage reflects this CG suppression (Table 1).

Strategy for codon replacement. Despite the low overall bias in codon usage in Sabin 2, some synonymous codons are used at much lower frequencies than others (Table 1). To examine codon usage in Sabin 2, we replaced the preferred codons for each of nine amino acids with a synonymous unpreferred codon (Table 1). The codon replacements were introduced only within the capsid sequences, because those sequences uniquely identify a poliovirus or enterovirus serotype (5, 16, 30, 62), as both noncapsid and 5′-UTR region sequences are exchanged out by recombination with other species C enteroviruses during poliovirus circulation (5, 16, 30, 62). Because codon usage bias was very low for most two-fold degenerate codons

(except codons for His and Tyr), we replaced only six-fold, four-fold, and three-fold degenerate codons. Synonymous codons for nine amino acids were replaced by a single unpreferred codon: CUU for Leu, AGC for Ser, CGG for Arg, CCG for Pro, GUC for Val, GCG for Ala, GGU for Gly, and AUC for Ile (Table 1). Whenever possible, we chose codons with G or C at degenerate positions, in order to increase the G + C content of the modified viral genomes.

Codon replacements were introduced into a full-length infectious cDNA clone derived from Sabin 2 (Fig. 1). The recombinant insert differed in sequence from the reference Sabin 2 strain (41, 45) at two synonymous third-position sites ($ACA_{2616}\rightarrow$ ACG and $CCA_{3303}\rightarrow$ CCT) modified to introduce unique EagI and XhoI restriction sites. Replacement codons were introduced into S2R9 within an interval (nt 748 to 3303) spanning all but the last 27 codons of the capsid region. The capsid interval was divided into four mutagenesis cassettes: A (nt 657 to 1317; 661 bp), B (nt 1318 to 2102; 785 bp), C (nt 2103 to 2615; 513 bp), and D (nt 2616 to 3302; 687 bp) (Fig. 1). Mutagenesis cassette A, bounded by restriction sites BstZ17I and AvrII, includes the last 91 bp of the 5′-untranslated region (5′-UTR), but no 5′-UTR sequences were modified in cassette A. Within each cassette, synonymous codons for the nine amino acids were comprehensively replaced except at positions where replacement would have generated an unwanted restriction site. Unmodified cassettes were identified by uppercase italic letters; the corresponding cassettes with modified codons were identified by lowercase italic letters. Thus, the reference Sabin 2 clone, S2R9, was identified as *ABCD*, and the fully modified construct (clone S2R23), was identified as *abcd* (Fig. 1).

The modifications dramatically altered the mono-, di-, and trinucleotide (codon) frequencies in the capsid region (Tables 1 and 2). In the fully modified construct, *abcd*, nearly half (427/879; 48.6%) of the capsid region codons were replaced, and a total of 544 substitutions

(90 first codon position, 44 second position, and 410 third position) were introduced into the 2555 mutagenized capsid region nucleotides (supplementary table). Compared with *ABCD*, the N_C values in the capsid region of *abcd* fell from 56.2 to 29.8, the number of CG dinucleotides rose from 97 to 302, and the %G + C increased from 48.4% to 56.4% (Table 2). These changes were nearly uniformly distributed over the mutagenized capsid region (Fig. 1; Table 2).

Growth properties of codon-replacement constructs. The growth properties of the virus constructs (Fig. 2) in cultured cells at 35°C were measured by plaque assays on HeLa cells (Fig. 3) and single-step growth experiments in HeLa cells (Fig. 4).

(i) Plaque assays. Describe experiment: Virus from in vitro transcripts transfected into HeLa cells, titered, and used to infect HeLa cells, plaque assays on HeLa cells (35°C 60 h). An approximately linear inverse relationship was observed between mean plaque area in HeLa cells and the number of nucleotide changes in the capsid region (Fig. 3). Similar inverse linear relationships were observed when the abscissa was rescaled to the number of replacement codons or to the number of CG dinucleotides (supplementary material). There was no strong polarity to the effects of codon replacement within the capsid region, as introduction of replacement codons into any combination of the four cassettes reduced plaque areas approximately in proportion to the total number of replacement codons. However, replacement of codons into VP1 (cassette D) appeared to have slightly stronger effects than replacement elsewhere (Fig. 3). Codon replacement in three or four cassettes generally conferred a minute-plaque phenotype (mean plaque area <25% that of the unmutagenized *ABCD* prototype), and the mean areas of the observed plaques of the *abcd* construct were ~9% of the *ABCD* prototype (Fig. 3). An exception was the *abcD* construct, which had a greater mean plaque area (~38% that of

the *ABCD* prototype) than the *Abcd*, *aBcd*, and *abCd* constructs, underscoring the stronger influence upon plaque size of codon replacement within VP1.

Measurement of plaque areas and total plaque number became increasingly difficult as plaque size decreased. The diameters of poliovirus plaques are typically heterogeneous, and this heterogeneity was observed with the plaques of all constructs. Precise measurement was most difficult with the smallest of the minute plaques, as was discriminating very minute plaques from other small defects in the cell monolayers. Extended incubation of plaque cultures to 72 h increased plaque diameters but did not markedly increase the plaque counts. Virus titers of all constructs were also determined by limit dilution infectivity assays in HEp-2(C) cells, yielding titers in good agreement with those obtained by plaque assay (data not shown).

(ii) Single-step growth experiments. A plaque is the result of several cycles of replication, which effectively amplifies any difference in replication rate. To examine the relationship between plaque size and virus yield, single-step growth experiments (input moi: 5 PFU/cell) were performed in HeLa cells at 35°C and the burst sizes were determined by plaque assay. Mean virus yields from the single-step growth experiments generally decreased as the number of replacement codons increased (Fig. 4). Virus yields were highest (~200 PFU/cell) for the *ABCD* prototype and constructs *ABcD* and *aBCD*. Yields were 4- to 8-fold lower with constructs *ABCd*, *abCD*, and *ABcd*, 12- to 24-fold lower with constructs *abcD* and *aBcd*, 30- to 45-fold lower with constructs *Abcd* and *abCd*, and ~65-fold lower with construct *abcd*. Maximum plaque yields were obtained at 10–12 h for all constructs (Fig. 4).

Physiological properties of codon-replacement constructs. Table: particle yields, particle/PFU ratios; Fig.: Intracellular viral proteins. Work in progress.

In vitro translation. Fig. Work in progress..

Predicted RNA secondary structures of codon-replacement construct genomes. M-FOLD analysis (34, 39, 58). Work in progress.

Stability of the mutant phenotypes. We examined the stability of the plaque and single-step growth yield phenotypes during serial passage in HeLa cells. Three constructs were studied: *ABCD* (unmodified prototype), *ABCd* (modified VP1 region), and *abcd* (modified P1/capsid region). Each virus was passaged 25 times (35°C for 36 h) in HeLa monolayers with the input moi varying from 0.1 to 0.4 PFU/cell; each passage represented at least two rounds of replication. At every fifth passage, virus plaque yields, plaque areas, and the genomic sequences of the bulk virus populations were determined, and the moi was readjusted to ~0.1 PFU/cell.

All three constructs evolved during serial passage, as measured by increasing plaque size, increasing virus yield, and changing genomic sequences (Table 3; Fig. 5). Evolution of the *ABCD* prototype was the least complex. Plaque areas increased ~6-fold from passage 0 to passage 15, and this was accompanied by nucleotide substitutions at 6 sites. By contrast, virus yields increased 2.5-fold over the 6 passage intervals. A $C_{1439}\rightarrow$ T substitution was fixed by passage 5, and the five other substitutions were fixed by passage 20. Mixed bases were found at passage 5 (C_{2609}>T), passage 10 (C_{3424}>T; G_{3586}>>A; G_{5501}>A) and passage 15 (A_{5630}>T). We found no evidence of back mutation or serial substitutions at a site. All substitutions mapped to the coding region, and 2 of 6 (33%) mapped to the capsid region, which represents 35.4% of the genome. In distinct contrast to the pattern of poliovirus evolution in humans, where the large majority of base substitutions generate synonymous codons (31), all six of the observed base substitutions (4 at the second codon position and 2 at the first codon position) generated amino acid replacements (Table 3). None of the substitutions involved loss of a CG dinucleotide.

Evolution of the codon-replacement constructs was much more complex and dynamic. In construct *ABCd*, 4 of the 8 (50%) variable positions mapped to VP1 (12.1% of genome), and 3 of these 4 mapped within the replacement-codon *d* interval (9.2% of genome) (Table 3). Substitutions at half of the positions involved the apparent loss of CG dinucleotides (3.7% of total genome), although in all instances the loss was incomplete. One *d* interval substitution ($G_{3120}\rightarrow$ A) eliminating a CG dinucleotide represented a back mutation to the original synonymous codon. A second *d* interval substitution ($G_{2780}\rightarrow$ A) reduced the frequency of a CG dinucleotide by HeLa passage 10, but the CG pair was largely restored by HeLa passage 25. Another substitution ($C_{3377}\rightarrow$ T), which resulted in the partial loss of a CG dinucleotide, mapped just downstream from the *d* interval. Two adjacent substitutions, mapping to positions 3808 and 3809 in 2A, resulted in a complex pattern of substitution involving first and second positions of the same codon, which may have generated a CGT codon as an intermediate. The *ABCd* construct resembled the *ABCD* prototype in that substitutions in 6 of the 8 generated amino acid replacements. By contrast, the *ABCd* construct differed markedly from the *ABCD* prototype because the dynamics of substitution had apparently not stabilized by passage 25, and mixed bases were found at all 8 positions of variability (Table 3). The active sequence evolution was accompanied by steadily increasing plaque areas over a ~6-fold range, while virus yields fluctuated over a narrow (~2-fold) range (Fig. 5).

Evolution of the *abcd* construct was the most dynamic, with 13 sites of variability. Most (11/13; 84.6%) of the variable sites mapped to the capsid region, all within the codon-replacement interval, 8 within VP1 and 3 within VP2 (Table 3). As with the other constructs, most (8/13; 61.5%) of the substitutions encoded amino acid replacements. Substitutions at 6 sites involved partial, transient, or complete loss of CG dinucleotides. As in the *ABCd* construct,

a G_{3120}→ A substitution eliminated a CG dinucleotide and restored the original Sabin 2 base. Interestingly, this same reversion was observed in 8 other independent passages of the *abcd* construct (data not shown). The two variable sites outside of the capsid region (one in 2A, the other in 2C) stabilized with new substitutions by HeLa passage 20, whereas 8 of the 11 variable sites within the capsid region still had mixed bases at passage 25. Apart from the site of reversion at position 3120, all other variable sites differed between the *ABCD*, *ABCd*, and *abcd* constructs. No net changes were observed at site A_{481} (in the 5′-UTR), and U_{2909} (in the VP1 region), known to be strongly selected against when Sabin 2 replicates in the human intestine (32, 35, 62, 63).

Most (9 of 13) of the capsid amino acid replacements mapped within or near surface determinants forming neutralizing antigenic sites. For example, four replacements mapped to site 1, four to site 2, and one to site 4 (Table 3). Although surface determinants are generally the most variable (55), amino acid replacements also occurred in naturally variable non-surface residues in VP1 (Lys>Glu) and $2A^{pro}$ (Ser>Arg). Most of the synonymous mutations mapped to conserved amino acid positions. However, several of the amino acid replacements, including 5 of the 6 in the *ABCD* construct, were substitutions to non-consensus residues (Table 3). Plaque areas increased ~15-fold from passage 0 to passage 15, and virus yields increased ~4-fold between passages 5 and 10 (Fig. 5).

DISCUSSION

Although the Sabin 2 OPV strain has a low codon usage bias, its replicative fitness in cell culture could be sharply reduced by replacement of preferred codons in the capsid region with synonymous unpreferred codons. The reduction in fitness, as measured by plaque area, was approximately proportional to the length of the interval containing replacement codons. Plaque areas were reduced by ~90% and virus burst yields by ~98% in the *abcd* construct, in which the replacement interval spanned nearly the entire capsid region. The fitness declines in the replacement codon constructs are not attributable to amino acid substitutions because all constructs encoded the same reference Sabin 2 polyprotein sequence.

The relative contributions of individual codon replacements, or of the nine different categories of codon replacement, are presently unknown. Only one substitution, $G_{3120} \rightarrow A$, a direct back mutation to the original sequence, was shared between derivatives of the *ABCd* and *abcd* constructs after serial passage. The 19 other independent substitutions found between the *ABCd* and *abcd* high-passage derivatives were associated with 12 different codon triplets. Codon replacement in VP1 appeared to have greater relative effects on replicative fitness than replacements in other capsid intervals, an observation confirmed in similar experiments with the wild poliovirus type 2 prototype strain, MEF-1 (R. Campagnoli, unpublished results), and reinforced by the finding that 8 of the 13 sites that varied upon serial passage of the *abcd* construct mapped to VP1.

Multiple synonymous capsid codon replacements appear to be necessary for substantial reductions in poliovirus fitness. In our initial experiments, replacement of 3 to 14 Arg codons in

VP1 (0.3% to 1.6% of capsid codons) with CGG [among the least preferred codons in poliovirus (55)] did not result in any apparent reduction in plaque areas. The requirement for multiple codon replacements for impairment of gene expression is consistent with the results of codon replacements in the phosphoglycerate kinase gene of yeast (17) and in the L1 and L2 capsid protein genes of bovine papillomavirus (64). By contrast, introduction of 1, 6, and 10 strongly unpreferred CUA-Leu codons into the 5′-region of the alcohol dehydrogenase gene of *Drosophila* resulted in significant reductions in enzyme activity (7).

Although fitness of the *ABCd* and *abcd* constructs increased during serial passage in HeLa cells, the virus yields of the *ABCd* and *abcd* derivatives were still below that of the unmodified *ABCD* construct. However, the selection experiments were complicated by the sequence and fitness evolution of the reference *ABCD* construct, along with the *ABCd* and *abcd* constructs, during passage in HeLa cells. It is known that Sabin 2 accumulates mutations during propagation in cell culture, but the substitutions observed here are distinct from those previously described (54). Perhaps more significantly, the substitutions accumulating in the *ABCd* and *abcd* derivatives during cell culture passage were distinct from those frequently selected during replication of the Sabin 2 strain in the human intestine, which is primarily associated with changes at two positions (G_{481}→ A in the 5′-UTR and Ile_{143}→ Thr in VP1) (41, 44, 63). It appears likely that multiple substitutions contribute to the observed phenotypes of the codon-deoptimized constructs, and that full reversion to increased replicative fitness is a multistep process involving changes at multiple sites whose individual selection coefficients may be small (7).

A combination of mechanisms may contribute to the observed reduction in fitness of the codon-deoptimized constructs. The depletion of the tRNA pools may result in ribosomal pausing

at unpreferred codons, which may have several different physiological effects including increased costs of translational proofreading (6), amino acid misincorporation (40), frameshifting (15), premature polypeptide chain termination (40), degradation of the RNA template (17), and possible disruption of the closely synchronized co-translational proteolytic processing of the poliovirus polyprotein (42).

The biological basis for CG suppression in RNA viruses is poorly understood (25). However, upon codon deoptimization, CG shifted from the least abundant dinucleotide in *ABCD* [181] to the most abundant in *abcd* [386]. In at least six mutational events during serial passage of the *ABCd* and *abcd* constructs, selection against CG dinucleotides was apparently sufficiently strong to overcome the general tendency for amino acid conservation in the poliovirus capsid, although most of the changes mapped in or near variable antigenic sites. The presence of additional CG dinucleotides was so destabilizing that it led to a broader distribution of mutational variants within the populations of *ABCd* and *abcd* derivatives.

It may be possible to further deoptimize the Sabin 2 capsid sequences. Additional [e.g., AUA (Ile), AAA (Lys), and CAU (His)] and redesigned [e.g., UCG (Ser)] codon substitutions, better matched to the least abundant tRNA genes in the human genome (21), may further impair translational efficiency and reduce replicative fitness. Additional CG dinucleotides may be incorporated into the ORF by uniform replacement of degenerate third-position bases with C when the first base of the next codon is G. Replacement of codons specifying conserved amino acids may further stabilize the reduced fitness phenotype, as only synonymous mutations are most likely to confer increased fitness. Although additional capsid substitutions may have measurable effects, we do not anticipate that the additional fitness losses to be as large as those already observed. Biological effects may be more dramatic if codon deoptimization is extended

to the noncapsid region, representing ~54% of the poliovirus ORF. However, comprehensive deoptimization of the entire poliovirus ORF may reduce fitness below the threshold of viability.

Modulation of RNA virus replicative fitness by codon deoptimization may have possible application for producing RNA viruses with stabilized phenotypes of reduced replicative fitness. If the observed fitness reductions in the codon-deoptimized constructs are the cumulative effects of many individual substitutions, full phenotypic reversion may require numerous mutational steps occurring over many replication cycles. Such constructs would likely be more stable than most RNA virus point mutants (57), and might have potential application to the genetic stabilization of live, attenuated RNA virus vaccines.

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1. **Akashi, H.** 2001. Gene expression and molecular evolution. Curr. Opin. Genet. Dev. **11**:660-666.
2. **Akashi, H., and A. Eyre-Walker.** 1998. Translational selection and molecular evolution. Curr. Opin. Genet. Dev. **8**:688-693.
3. **André, S., B. Seed, J. Eberle, W. Schraut, A. Bültmann, and J. Haas.** 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J. Virol. **72**:1497-1503.
4. **Bennetzen, J., and B. Hall.** 1982. Codon selection in yeast. J. Biol. Chem. **257**:3026-3031.
5. **Brown, B. A., M. S. Oberste, K. Maher, and M. Pallansch.** 2003. Complete genomic sequencing shows that polioviruses and members of human enterovirus species C are closely related in the non-capsid coding region. J. Virol. **77**:8973-8984.
6. **Bulmer, M.** 1991. The selection-mutation-drift theory of synonymous codon usage. Genetics **129**:897-907.
7. **Carlini, D. B., and W. Stephan.** 2003. In vivo introduction of unpreferred synonymous codons into the Drosophila Adh gene results in reduced levels of ADH protein. Genetics **163**:239-243.
8. **Chiapello, H., F. Lisacek, M. Caboche, and A. Henaut.** 1998. Codon usage and gene function are related in sequences of Arabidopsis thaliana. Gene. **209**:GC1-GC38.
9. **Chumakov, K. M.** 1996. PCR engineering of viral quasispecies: a new method to preserve and manipulate genetic diversity of RNA virus populations. J. Virol. **70**:7331-7334.
10. **Domingo, E., E. Baranowski, C. Escarmís, F. Sobrino, and J. J. Holland.** 2002. Error frequencies of picornavirus RNA polymerases: evolutionary implications for virus populations, p. 285-298. *In* B. L. Semler and E. Wimmer (ed.), Molecular Biology of Picornaviruses. ASM Press, Washington, D.C.
11. **Dominguez, G., C. Y. Wang, and T. K. Frey.** 1990. Sequence of the genome RNA of rubella virus: evidence for genetic rearrangement during togavirus evolution. Virology **177**:225-38.
12. **Drake, J. W., and J. J. Holland.** 1999. Mutation rates among RNA viruses. Proc. Natl. Acad. Sci. USA **96**:13910-13913.
13. **Duret, L.** 2002. Evolution of synonymous codon usage in metazoans. Curr. Opin. Genet. Dev. **12**:640-649.
14. **Estes, M. K.** 2001. Rotaviruses and their replication, p. 1747-1785. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia.
15. **Gallant, J., P. Bonthuis, and D. Lindsley.** 2003. Evidence that the bypassing ribosome travels through the coding gap. Proc. Natl. Acad. Sci. USA **100**:13430-13435.
16. **Georgescu, M. M., F. Delpeyroux, and R. Crainic.** 1995. Tripartite genome organization of a natural type 2 vaccine/nonvaccine recombinant poliovirus. J. Gen. Virol. **76**:2343-2348.

17. **Hockema, A., R. A. Kastelein, M. Vasser, and H. A. de Boer.** 1987. Codon replacement in the *PGK1* gene of *Saccharomyces cerevisiae*: experimental approach to study the role of biased codon usage in gene expression. Mol. Cell Biol. **7**:2914-2924.

18. **Hughes, P. J., D. M. Evans, P. D. Minor, G. C. Schild, J. W. Almond, and G. Stanway.** 1986. The nucleotide sequence of a type 3 poliovirus isolated during a recent outbreak of poliomyelitis in Finland. J. Gen. Virol. **67**:2093-2102.

19. **Ikemura, T.** 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. J. Mol. Biol. **146**:1-21.

20. **Ikemura, T.** 1982. Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and *Escherichia coli* with reference to the abundance of isoaccepting transfer RNAs. J. Mol. Biol. **158**:573-597.

21. **International Human Genome Sequencing Consortium.** 2001. Initial sequencing and analysis of the human genome. Nature **409**:860-921.

22. **Jenkins, G. M., and E. C. Holmes.** 2003. The extent of codon usage bias in human RNA viruses and its evolutionary origin. Virus Res. **92**:1-7.

23. **Kanaya, S., Y. Yamada, M. Kinouchi, Y. Kudo, and T. Ikemura.** 2001. Codon usage and tRNA genes in eukaryotes: correlation of codon usage diversity with translation efficiency and with CG-dinucleotide usage as assessed by multivariate analysis. J. Mol. Evol. **53**:290-298.

24. **Kane, J. F.** 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli. Current Opinion in Biotechnology **6**:494-500.

25. **Karlin, S., W. Doerfler, and L. R. Cardon.** 1994. Why is CpG suppressed in the genomes of virtually all small eukaryotic viruses but not in those of large eukaryotic viruses? J. Virol. **68**:2889-2897.

26. **Kew, O. M., V. Morris-Glasgow, M. Landaverde, C. Burns, J. Shaw, Z. Garib, J. André, E. Blackman, C. J. Freeman, J. Jorba, R. Sutter, G. Tambini, L. Venczel, C. Pedreira, F. Laender, H. Shimizu, T. Yoneyama, T. Miyamura, H. van der Avoort, M. S. Oberste, D. Kilpatrick, S. Cochi, M. Pallansch, and C. de Quadros.** 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science **296**:356-359.

27. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structrure, gene organization and polypeptide expression of poliovirus RNA. Nature **291**:547-553.

28. **Kohara, M., T. Omata, T. Kameda, B. L. Semler, H. Itoh, E. Wimmer, and A. Nomoto.** 1985. In vitro phenotypic markers of a poliovirus recombinant constructed from infectious cDNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J. Virol. **53**:786-792.

29. **La Monica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515-525.

30. **Liu, H.-M., D.-P. Zheng, L.-B. Zhang, M. S. Oberste, O. M. Kew, and M. A. Pallansch.** 2003. Serial recombination during circulation of type 1 wild-vaccine recombinant polioviruses in China. J. Virol. **77**:10994-11005.

31. **Liu, H.-M., D.-P. Zheng, L.-B. Zhang, M. S. Oberste, M. A. Pallansch, and O. M. Kew.** 2000. Molecular evolution of a type 1 wild-vaccine poliovirus recombinant during widespread circulation in China. J. Virol. **74:**11153-11161.

32. **Macadam, A. J., S. R. Pollard, G. Ferguson, R. Skuce, D. Wood, J. W. Almond, and P. D. Minor.** 1993. Genetic basis of attenuation of the Sabin type 2 vaccine strain of poliovirus in primates. Virology **192:**18-26.

33. **Martín, J., G. L. Ferguson, D. J. Wood, and P. D. Minor.** 2000. The vaccine origin of the 1968 epidemic of type 3 poliomyelitis in Poland. Virology **278:**42-49.

34. **Mathews, D. H., J. Sabina, M. Zuker, and D. H. Turner.** 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. **288:**911-940.

35. **Minor, P. D., and G. Dunn.** 1988. The effect of sequences in the 5′ non-coding region on the replication of polioviruses in the human gut. J. Gen. Virol. **69:**1091-1096.

36. **Moriyama, E. N., and J. Powell.** 1997. Codon usage bias and tRNA abundance in *Drosophila*. J. Mol. Evol. **45:**514-523.

37. **Musto, H., S. Cruvellier, G. D'Onofrio, H. Romero, and G. Bernardi.** 2001. Translational selection on codon usage in *Xenopus laevis*. Mol. Biol. Evol. **18:**1703-1707.

38. **Osawa, S., T. H. Jukes, K. Watanabe, and A. Muto.** 1992. Recent evidence for evolution of the genetic code. Microbiol. Rev. **56:**229-264.

39. **Palmenberg, A. C., and J.-Y. Sgro.** 1997. Topological organization of picornaviral genomes: statistical prediction of RNA structural signals. Semin. Virol. **8:**231-241.

40. **Parker, J.** 1989. Errors and alternatives in reading the universal genetic code. Microbiol. Rev. **53:**273-298.

41. **Pollard, S. R., G. Dunn, N. Cammack, P. D. Minor, and J. W. Almond.** 1989. Nucleotide sequence of a neurovirulent variant of the type 2 oral poliovirus vaccine. J. Virol. **63:**4949-4951.

42. **Racaniello, V. R.** 2001. *Picornaviridae:* the viruses and their replication, p. 685-722. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia.

43. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78:**4887-4891.

44. **Ren, R., E. G. Moss, and V. R. Racaniello.** 1991. Identification of two determinants that attenuate vaccine-related type 2 poliovirus. J. Virol. **65:**1377-1382.

45. **Rezapkin, G. V., L. Fan, D. M. Asher, M. R. Fibi, E. M. Dragunsky, and K. M. Chumakov.** 1999. Mutations in Sabin 2 strain of poliovirus and stability of attenuated phenotype. Virology **258:**152-160.

46. **Robinson, M., R. Lilley, S. Little, J. S. Emtage, G. Yarronton, P. Stephens, A. Millican, M. Eaton, and G. Humphreys.** 1984. Codon usage can affect efficiency of translation of genes in *Escherichia coli*. Nucleic Acids Res. **12:**6663-6671.

47. **Rothberg, P. G., and E. Wimmer.** 1981. Mononucleotide and dinucleotide frequencies, and codon usage in poliovirion RNA. Nucleic Acids Res. **9:**6221-6229.

48. **Rueckert, R. R., and M. A. Pallansch.** 1981. Preparation and characterization of encephalomyocarditis (EMC) virus. Meth. Enzymol. **78:**315-325.

49. **Sabin, A. B., and L. R. Boulger.** 1973. History of Sabin attenuated poliovirus oral live vaccine strains. J. Biol. Stand. **1**:115-118.

50. **Sambrook, J., and D. W. Russell.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

51. **Smith, D. W.** 1996. Problems of translating heterologous genes in expression systems: the role of tRNA. Biotech. Prog. **12**:417-422.

52. **Stemmer, W. P. C., A. Crameri, K. D. Ha, T. M. Brennan, and H. L. Heyneker.** 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene **164**:49-53.

53. **Sutter, R. W., O. M. Kew, and S. L. Cochi.** 2003. Poliovirus vaccine -- live, p. 651-705. *In* S. A. Plotkin and W. A. Orenstein (ed.), Vaccines, Fourth ed. W.B. Saunders Company, Philadelphia.

54. **Taffs, R. E., K. M. Chumakov, G. V. Rezapkin, Z. Lu, M. Douthitt, E. M. Dragunsky, and I. S. Levenbook.** 1995. Genetic stability and mutant selection in Sabin 2 strain of oral poliovirus vaccine grown under different cell culture conditions. Virology **209**:366-373.

55. **Toyoda, H., M. M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes: Implication for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561-585.

56. **Urrutia, A. O., and L. D. Hurst.** 2001. Codon usage bias covaries with expression breadth and the rate of synonymous evolution in humans, but this is not evidence for selection. Genetics **159**:1191-1199.

57. **Wimmer, E., C. U. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Ann. Rev. Genet. **27**:353-436.

58. **Witwer, C., S. Rauscher, I. L. Hofacker, and P. F. Stadler.** 2001. Conserved secondary structures in *Picornaviridae* genomes. Nucleic Acids Res. **29**:5079-5089.

59. **Wright, F.** 1990. The effective number of codons used in a gene. Gene **87**:23-29.

60. **Xia, X.** 1998. How optimized is the translational machinery in *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*? Genetics **149**:37-44.

61. **Yadava, A., and C. F. Ockenhouse.** 2003. Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems. Infect. Immun. **71**:4961-4969.

62. **Yang, C.-F., T. Naguib, S.-J. Yang, E. Nasr, J. Jorba, N. Ahmed, R. Campagnoli, H. van der Avoort, H. Shimizu, T. Yoneyama, T. Miyamura, M. A. Pallansch, and O. Kew.** 2003. Circulation of endemic type 2 vaccine-derived poliovirus in Egypt, 1983 to 1993. J. Virol. **77**:8366-8377.

63. **Yoshida, H., H. Horie, K. Matsuura, T. Kitamura, S. Hashizume, and T. Miyamura.** 2002. Prevalence of vaccine-derived polioviruses in the environment. J. Gen. Virol. **83**:1107-1101.

64. **Zhou, J., W. J. Liu, S. W. Peng, X. Y. Sun, and I. Frazer.** 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. J. Virol. **73**:4972-4982.

Table 1. Codon usage in mutagenized capsid interval and complete open reading frame in both unmodified and deoptimized Sabin 2 genomes

Amino acid	Codon[a]	Codon usage (number)			
		Capsid interval (nt 748 to 3303)		Complete ORF (nt 748 to7368)	
		Construct		Construct	
		ABCD[b]	*abcd*[c]	*ABCD*	*abcd*
Leu	UUA	9	1	25	17
	UUG	18	2	40	24
	CUU	4	55	22	73
	CUC	7	0	27	20
	CUA	7	1	33	27
	CUG	14	0	25	11
Ser	UCU	8	0	19	11
	UCC	14	2	33	21
	UCA	18	0	43	25
	UCG	6	0	8	2
	AGU	10	0	26	16
	AGC	9	63	20	74
Arg	CGU	0	0	3	3
	CGC	11	0	13	2
	CGA	4	0	7	3
	CGG	2	39	7	44
	AGA	17	0	45	28
	AGG	5	0	23	18
Pro	CCU	12	2	18	8
	CCC	19	0	32	13
	CCA	21	0	53	32
	CCG	9	59	19	69
Thr	ACU	20	0	47	27
	ACC	24	1	55	32
	ACA	20	0	47	27
	ACG	11	74	17	80
Val	GUU	17	0	40	23
	GUC	10	55	21	66
	GUA	10	1	24	15
	GUG	20	1	55	36
Ala	GCU	19	0	49	30
	GCC	16	2	40	26
	GCA	23	0	61	38
	GCG	10	66	17	73

Table 1. (continued)

Gly	**GGU**	14	52	42	80
	GGC	8	0	30	22
	GGA	12	0	38	26
	GGG	20	2	37	19
Ile	AUU	14	0	59	45
	AUC	15	45	47	77
	AUA	16	0	30	14
Phe	UUU	21	21	48	48
	UUC	14	14	36	36
Tyr	UAU	16	16	43	43
	UAC	21	21	57	57
Cys	UGU	5	5	22	22
	UGC	10	10	20	20
His	CAU	6	6	19	19
	CAC	12	12	30	30
Gln	CAA	18	18	47	47
	CAG	9	9	32	32
Asn	AAU	25	25	52	52
	AAC	25	25	61	61
Lys	AAA	13	13	64	64
	AAG	18	18	58	58
Asp	GAU	19	19	62	62
	GAC	23	23	51	51
Glu	GAA	16	16	57	57
	GAG	19	19	56	56
Trp	UGG	13	13	28	28
Met	AUG	26	26	67	67

[a] Unpreferred codons used for codon deoptimization are shown in boldface font

[b] *ABCD* is construct S2R9, which differs from the reference Sabin 2 strain sequence only at two synonymous third-position sites

[c] *abcd* is construct S2R23, which has replacement codons across an interval spanning 97% of the capsid region

Table 2. Effective number of codons used (N_C), number of CG dinucleotides, and G+C content in mutagenized capsid region sequences

Construct[a]	Length of mutagenized interval (bp)	N_C[b]			No. of CG dinucleotides[c]			%G+C		
		Muta-genized interval	Complete capsid region[d]	Complete ORF	Muta-genized interval	Complete capsid region	Complete ORF	Muta-genized interval	Complete capsid region	Complete ORF
ABCD	2555[e]	56.0[f]	56.2	54.6	94	97	181	48.5	48.4	46.0
aBCD	570[e]	30.8	56.1	56.3	63	140	224	56.0	50.1	46.7
AbCD	785	29.9	53.1	55.7	89	161	245	56.1	50.7	47.0
ABcD	513	28.2	56.3	56.0	59	143	227	57.0	50.1	46.7
ABCd	687	28.4	54.6	56.5	88	149	233	57.7	50.7	46.5
abcd	2555	29.3	29.8	47.3	299	302	386	56.7	56.4	49.2

[a] Constructs corresponded to the following recombinant plasmids: *ABCD*, S2R9; *aBCD*, S2R28; *AbCD*, not constructed; *ABcD*, S2R20; *ABCd*, S2R19; *abcd*, S2R23; N_C, number of CG dinucleotides, and %G+C of all other constructs can be calculated from table.

[b] N_C: effective number of codons used (1); one deoptimized codon spanned the EagI restriction cleavage site and was counted as part of Fragment D.

[c] One CG dinucleotide spanned the EagI restriction cleavage site and was counted as part of the Fragment D.

[d] Complete capsid region: nt 748 to 3384.

[e] Does not include terminal 91 bases of 5′-UTR at 5′-end of Fragment A (nt 657 to 747) that were not mutagenized.

[f] All values for mutagenized interval for *ABCD* construct represent baseline data for unmutagenized capsid interval targeted for codon-deoptimization (nt 748 to 3302).

1. **Wright, F.** 1990. The effective number of codons used in a gene. Gene **87:**23-29.

Table 3. Nucleotide substitutions in *ABCD*, *ABCd*, and *abcd* constructs during passage in Hela cells

Construct[a]	Nt position	Nucleotide substitutions						−1 nt[b]	Codon change[c,d,e]	+4 nt[b]	Amino acid subst.[d]	Gene	Location in Polyprotein[f]
		RD1	HeLa5	HeLa10	HeLa15	HeLa20	HeLa25						
ABCD	1439	U	C>U	C	C	C	C	C	CUU→ CCU	G	L→ P	VP2	S: NAg-2
	2609	C	C>U	U	U	U	U	U	GCA→ GUA	U	A→ V	VP1	I: NC
	3424	U	U>C	C>>U	C	C	C	C	UAC→ CAC	A	Y→ H	2A	NC
	3586	A	A	G>>A	G	G	G	G	AGA→ GGA	A	R→ G	2A	NC
	5501	A	A	G>A	G	G	G	C	AAA→ AGA	G	K→ R	3C	NC
	5630	A	A	A	A>U	U	U	U	CAG→ CUG	G	Q→ L	3C	NC
ABCd	1456	A	A>>G	A>>G	A>G	A=G	G>A	U	AAC→ GAC	C	N→ D	VP2	S: NAg-2
	2776	A	A	A	A>G	A>G	A>G	G	AAG→ GAG	C	K→ E	VP1	S: NAg-1
	2780	G	G>>A	A>G	G>A	G=A	G>A	G	CGG↔CAG	G	R↔Q	VP1	S: NAg-1
	3120[g]	G	G	G	G>A	A>G>>C	A>C>>G	U	GCG→ GCA	A	A	VP1	I: C
	3377	C	C	C	C>U	C>U	C>U	A	ACG↔AUG	A	T↔M	VP1	I: NC
	3808	U	U	U	U>C	U>C	U>>C	U	UAU→ UGU	G	Y→ R	2A	NC
	3809	A	A>G	G>>A	G=A	G>A	G>>A						
	4350	A	A>G	G>A	G=A	G>A	G=A	C	UUA↔UUG	U	L	2C	C
abcd	1169	G	G	G>>A	A>>G	G>A	G>A	G	CGG↔CAG	A	R↔Q	VP2	S: NAg-4
	1447	A	A	A	A	A=G	G>A	G	AAC→ GAC	G	N→ D	VP2	S: NAg-2
	1608	U	U	U	U	U=C	C>U	C	GAU→ GAC	A	D	VP2	I: C
	2622	C	C	C>>U	U>>C	C>U	C	C	GUC↔GUU	G	V	VP1	I: C
	2633	C	C	C	U>>C	C>>U	C	U	GCG↔GUG	A	A↔V	VP1	I: NC
	2903	A	A	A	A	A=G	G>A	C	AAC→ AGC	U	N→ S	VP1	S: NAg-1
	2915	C	C	C>U	C>>U	C>U	C>>U	U	GCG↔GUG	A	A↔V	VP1	~S: ~NAg-1
	2986	A	A	A	A	A=G	G>A	U	AAA→ GAA	U	K→ E	VP1	I: V
	3120[g]	G	G>A	G=A	A>>G	A>>G	A>>G	U	GCG→ GCA	A	A	VP1	I: NC
	3121	A	A	A	A>>C	A>C	A>C	G	AAA→ CAA	G	K→ Q	VP1	I: C
	3150	G	G	G	A>G	G	G	C	ACG→ ACA	G	T	VP1	S: NAg-2
	3480	U	U>G	G>U	G>>U	G	G	G	AGU→ AGG	G	S→ R	2A	V
	4473	G	G	G	A>G	A	A	C	AAG→ AAA	C	K	2C	C

Footnotes to Table 3

[a] Constructs: *ABCD*, S2R9; *ABCd*, S2R19; *abcd*, S2R23.

[b] Nucleotides immediately preceding (–1 nt) and immediately following (+4 nt) codon

[c] Varying nucleotide is shown in boldface

[d] Rightward pointing arrows indicate substitutions that steadily accumulated with increased passage; bidirectional arrows indicate bidirectional fluctuations among substitutions

[e] CG dinucleotides, including those across codons, are underlined

[f] Location of amino acid replacements: S, virion surface residue; NAg, neutralizing antigenic site; ~NAg, adjacent to neutralizing antigenic site; I, internal capsid residue not exposed to virion surface; NC, non-consensus amino acid; V, variable amino acid

[g] Represents direct reversion of engineered codon change

Figure legends

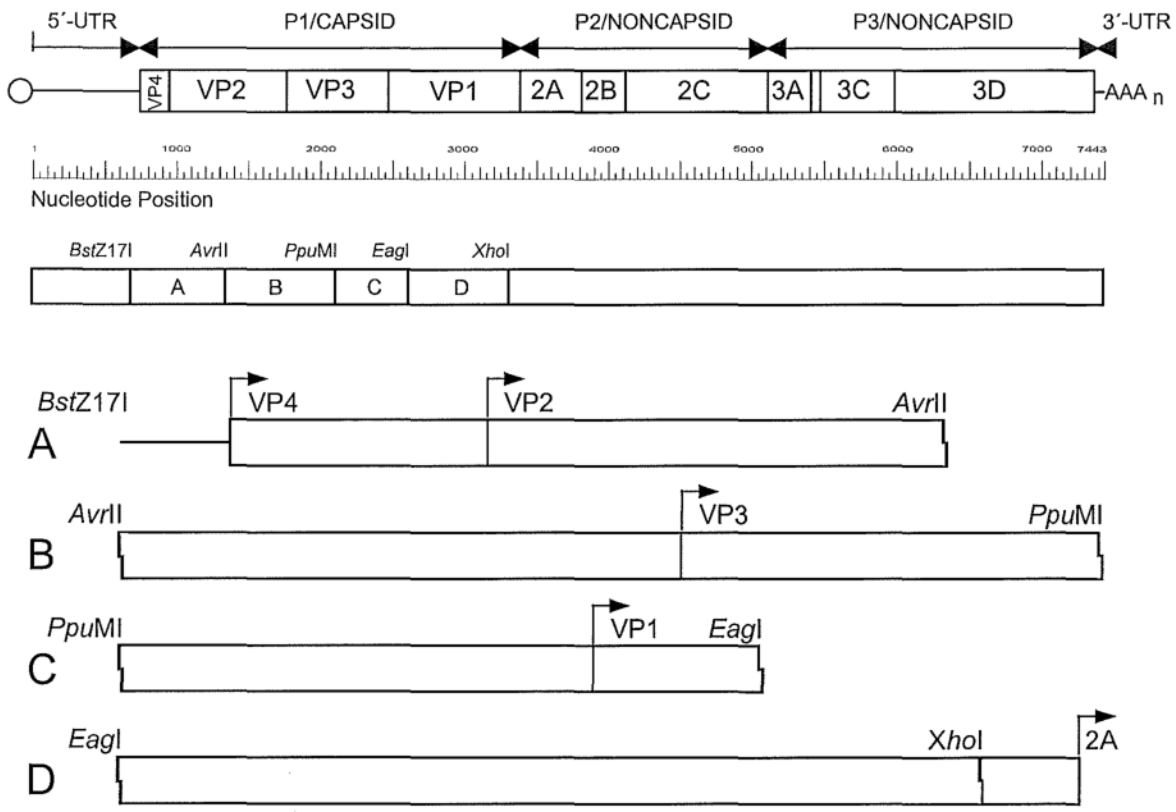
FIG. 1. A. Locations of the codon replacement cassettes A-D in the infectious Sabin 2 (S2R9) cDNA clone. The single open reading frame (ORF) is represented by an open rectangle, flanked by the 5′ and 3′ untranslated regions (UTRs). The restriction sites used for construction of the codon replacement constructs are indicated at the appropriate positions, in the context of the mature viral proteins. Colored bars identify replacement codon positions. Location CG dinucleotides (original, gray above; new, black below) are indicated by asterisks. B. Deoptimized codons of cassette D. Original S2R9 Sabin 2 triplets are shown above the codon-replacement residues; the deduced amino acids for both the *D* and *d* constructs are indicated above the triplets. Original CG dinucleotides are shown in green, new CG dinucleotides are shown in blue, CG dinucleotides lost during codon-deoptimization are shown in red.

FIG. 2. Sabin 2 codon replacement constructs. The Sabin 2 genome is represented with open rectangles. Filled rectangles indicate the locations of individual cassettes, black-filled rectangles indicate cassettes with replacement codons. Unmodified cassettes are indicated by upper case letters; the corresponding cassettes with replacement codons are indicated by lower case letters.

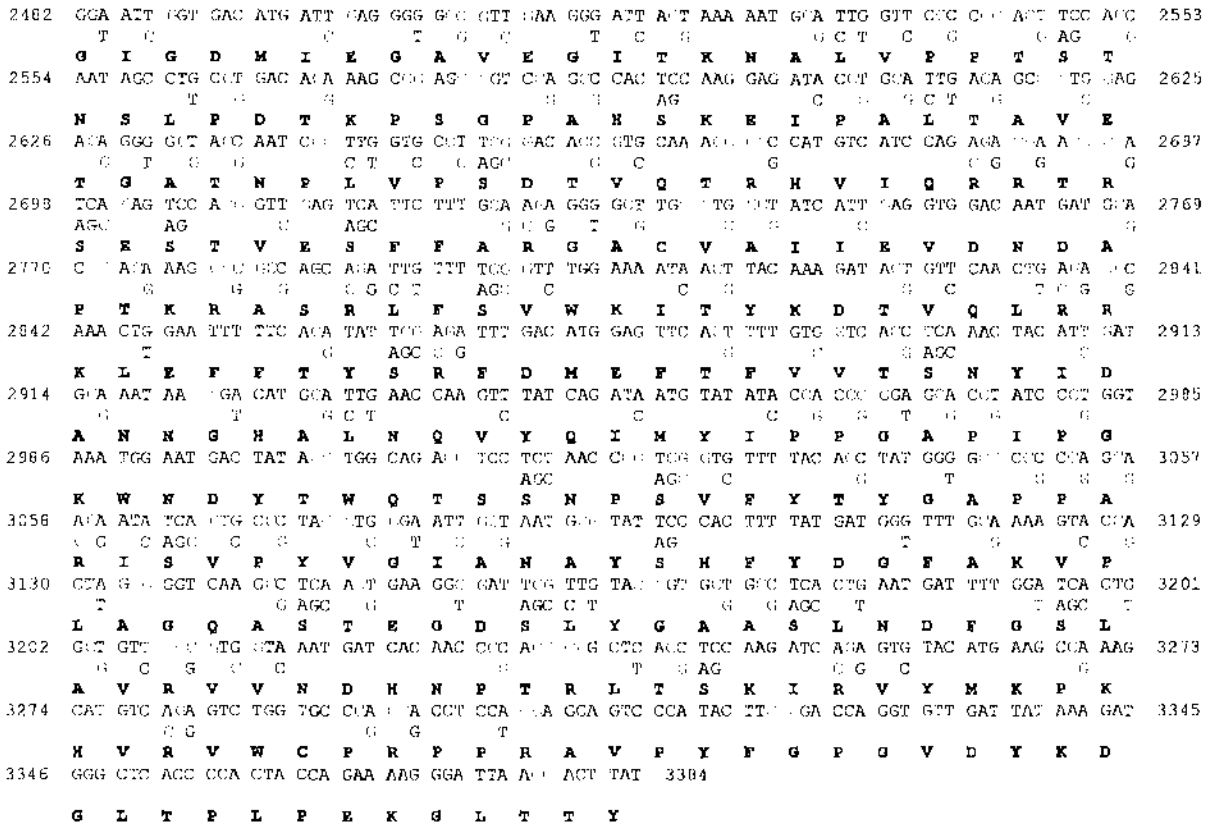
FIG. 3. A. (upper left) Mean plaque area in HeLa cells versus the number of nucleotide substitutions in the capsid region. Mean plaque areas were determined for plaques on monolayer HeLa cells after 52 hours incubation at 35°C. The coefficient of determination (R^2) for the regression line was 0.88. B. (upper right) Virus yields (12-hour postinfection) of a single-step growth curve versus the number of nucleotide substitutions in the capsid region. The coefficient of determination (R^2) for the regression line was 0.94. C. (lower) Plaque phenotypes at 35°C in HeLa cells.

FIG. 4. Single-step growth curves in HeLa S3 cells at 35°C. Single-step growth curves were performed as described in Materials and Methods, and burst sizes were determined by plaque assay of HEp-2C cells at 35°C.

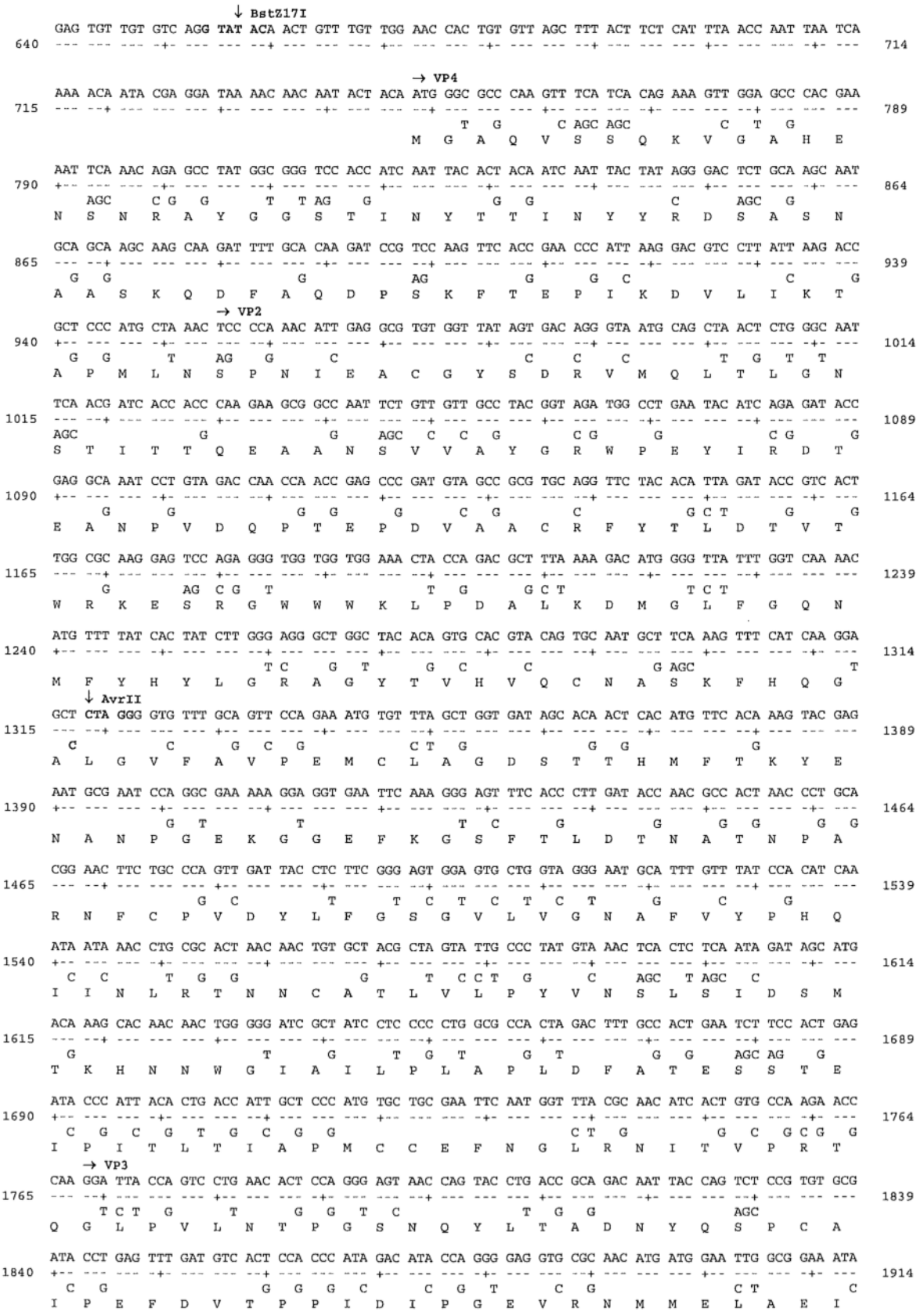
FIG. 5. Virus passage in HeLa cells at 35°C. A. (upper left) Mean plaque areas of evolving viruses were determined by plaque assay of HeLa cells after 52 hours incubation at 35°C. B. (upper right) Virus titers were determined by plaque assay of HeLa cells at 35°C on every fifth passage. C. (lower) Plaque phenotypes at 35°C in HeLa cells.

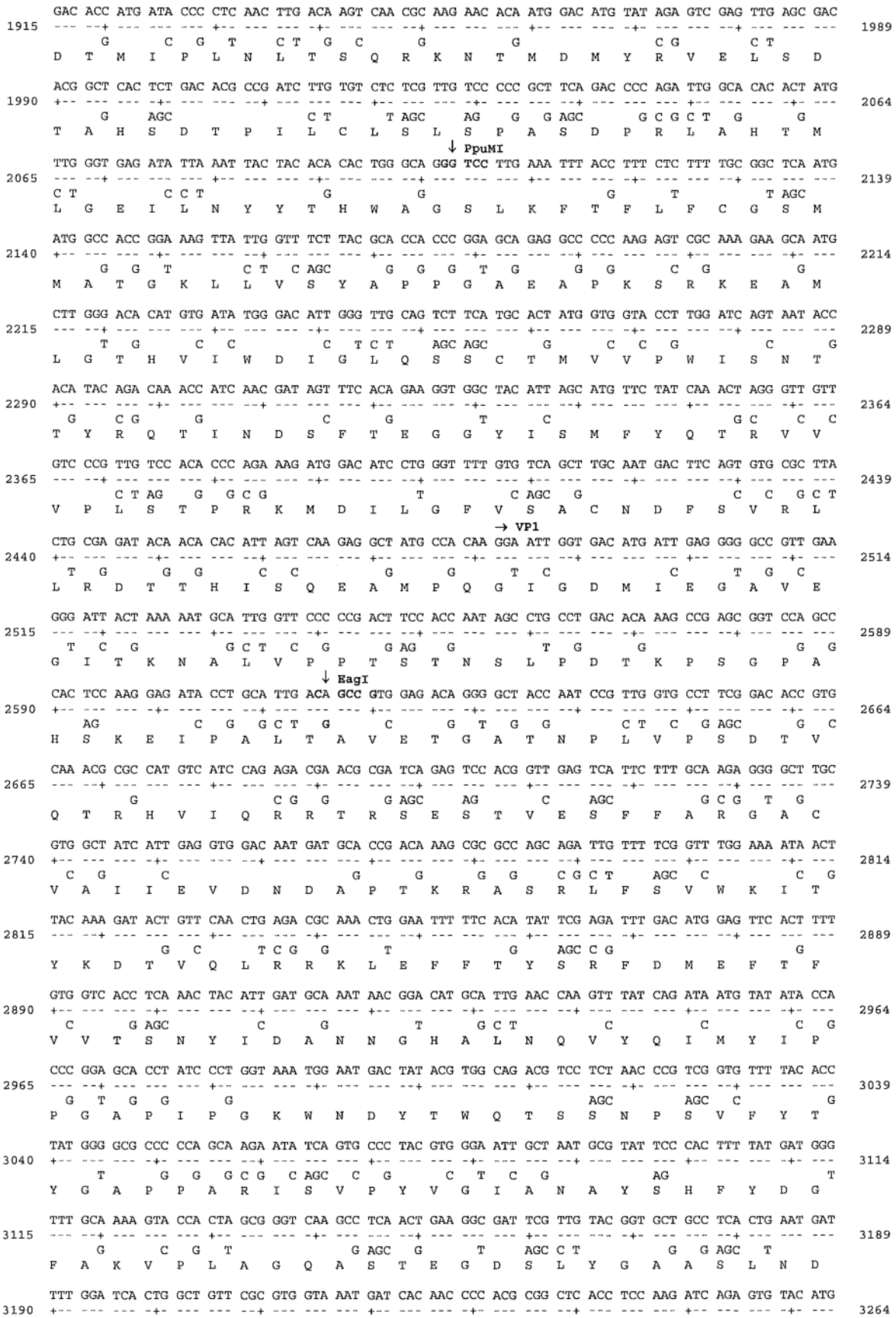


Burns *et al.*: Figure 1A



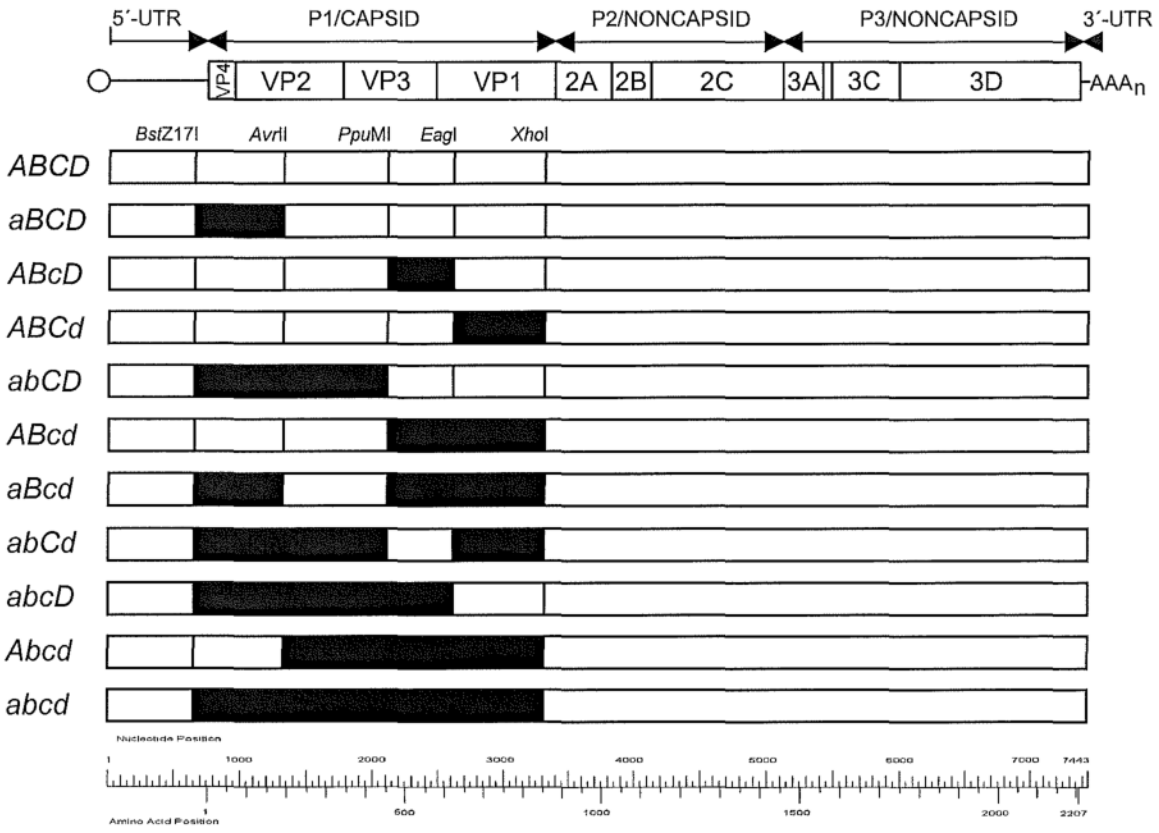
Burns et al.: Figure 1B


↓ BstZ17I
→ VP4
→ VP2
↓ AvrII
→ VP3

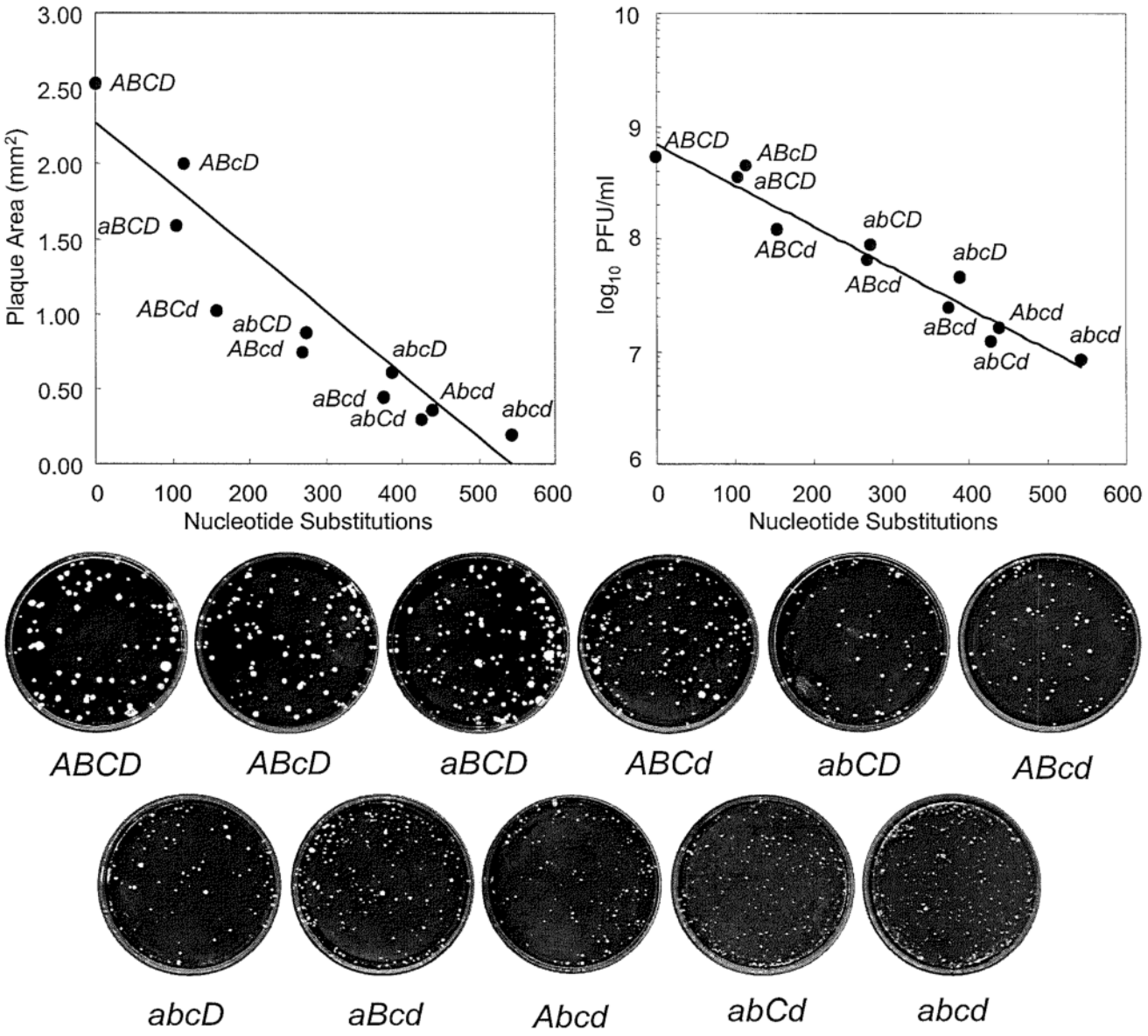

PpuMI
VP1
EagI

```
        T AGC   T   G   C   G   C   C                   G           T   G AG         C G   C
    F   G   S   L   A   V   R   V   V   N   D   H   N   P   T   R   L   T   S   K   I   R   V   Y   M
                                                        ↓ XhoI
    AAG CCA AAG CAT GTC AGA GTC TGG TGC CCA CGA CCT CCA CGA GCA GTC CCA TAC TTC GGA CCA GGT GTT GAT TAT
3265 --- --+ --- --- --- +-- --- --- -+- --- --- --+ --- --- --- +-- --- --- -+- --- --- --+ --- --- ---  3339
      G             C G                 G   G       T
    K   P   K   H   V   R   V   W   C   P   R   P   P   R   A   V   P   Y   F   G   P   G   V   D   Y

    AAA GAT GGG CTC ACC CCA CTA CCA GAA AAG GGA TTA ACG ACT TAT
3340 +-- --- --- -+- --- --- --+ --- --- --- +-- --- --- -+- ---  3384

    K   D   G   L   T   P   L   P   E   K   G   L   T   T   Y
```

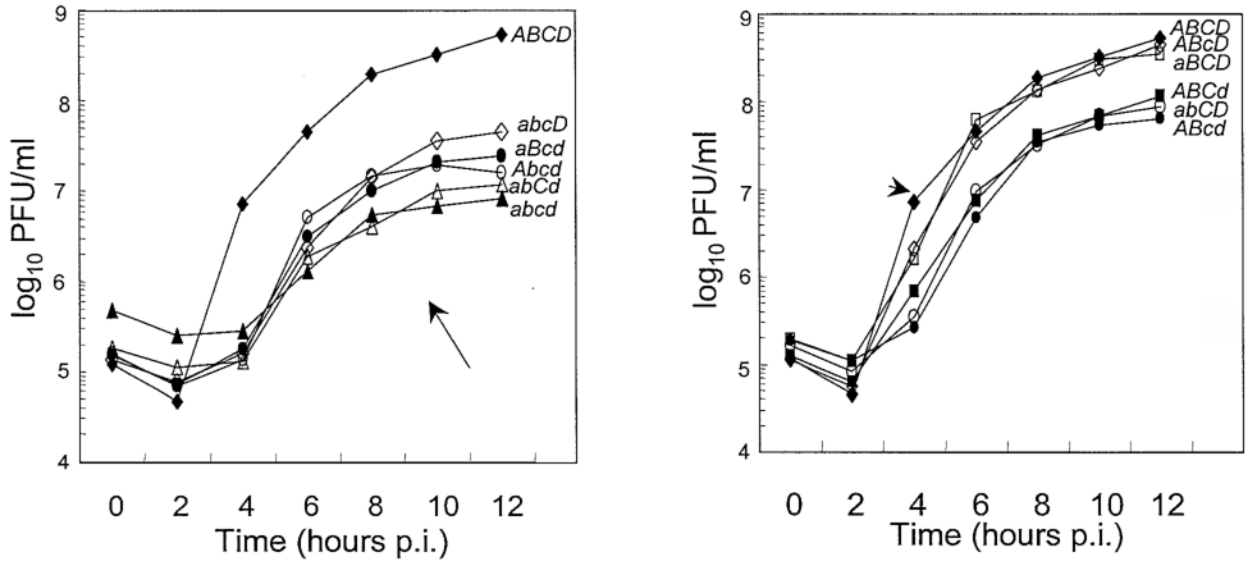
BURNS et al.: SUPPLEMENTARY FIGURE
TOTAL DEOPTIMIZED CODONS



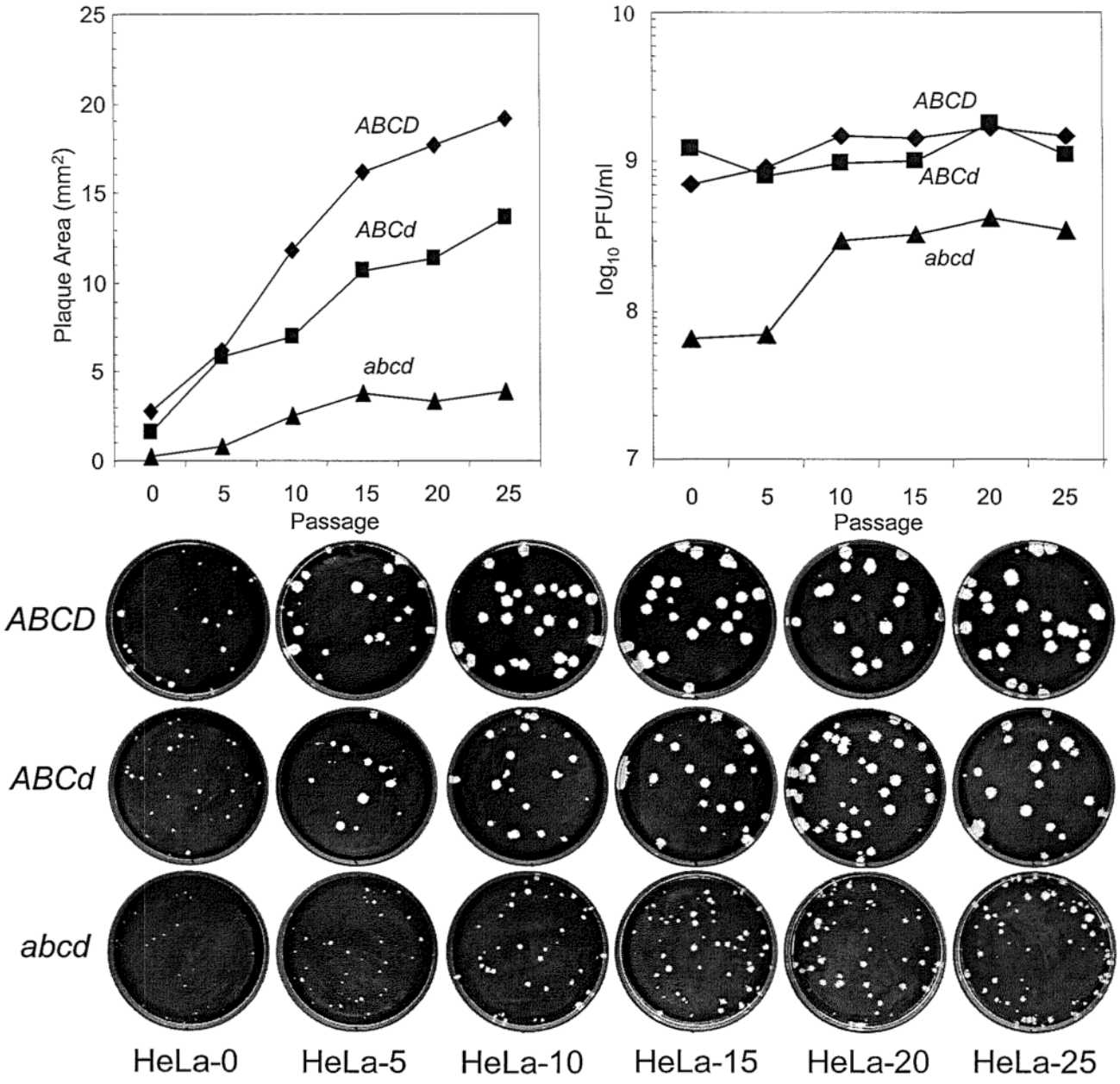
Burns *et al.*: Figure 2



Burns *et al.*: Figure 3



Burns *et al.*: Figure 4



Burns *et al.*: Figure 5