

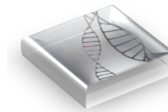
Rescuing Ebola Makona Using Reverse Genetics and SMRT Sequencing

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Center for Genome Sciences

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“Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.”



Overview

1. Ebola Virus



<http://www.md-health.com>

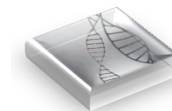
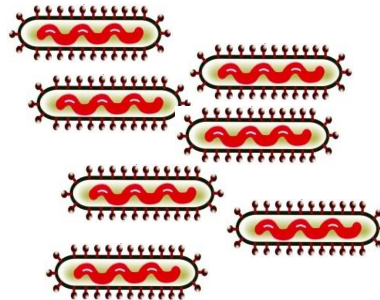
2. RNA Reverse Genetics

3. PacBio SMRT Sequencing



<http://www.pacb.com>

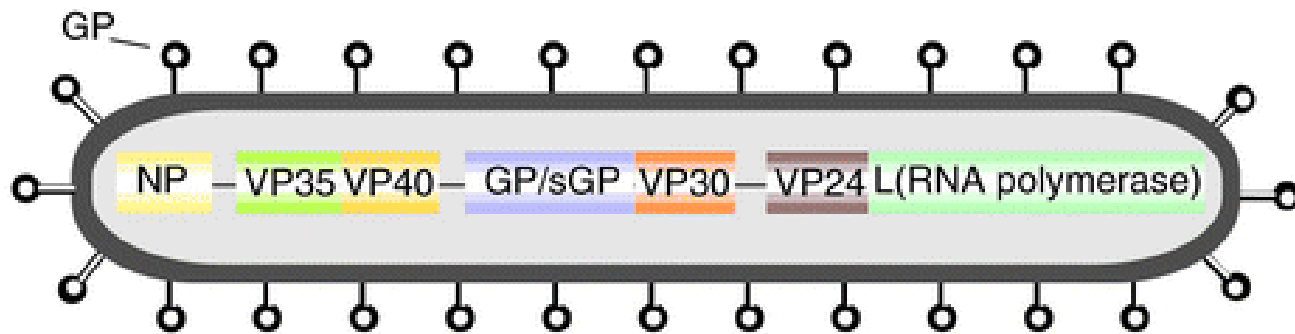
4. Virus Rescue



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Ebola Background

- Discovered in 1976
- Filovirus (genus *Ebolavirus*)
 - **Zaire**, Sudan, Tai Forest, Bundibugyo, Reston
- Single-strand negative sense
- RNA of ~19 kilobases
- Produces 7 mRNAs upon infection

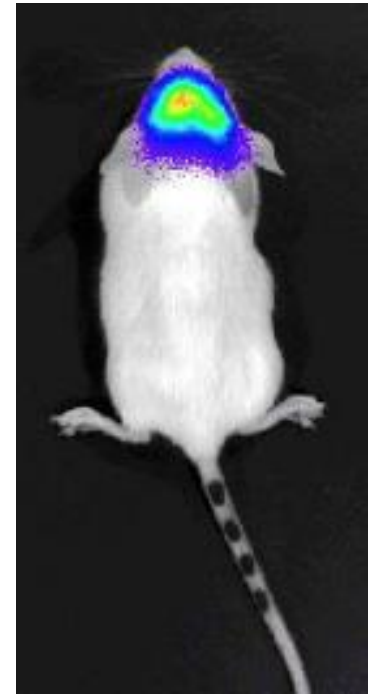


Takada A and Kawaoka Y. Trends Microbiol. 2001 Oct;9(10):5D6-11

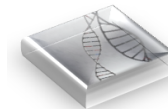
Reverse Genetics of RNA Viruses

Generation of replication competent virus entirely from cDNA

- Recover viruses:
 - Which only sequence information exists
 - Isolates that are nearly impossible to acquire
- Tag virus (eg. GFP, luciferase)



<http://jvi.asm.org/content/77/9/5333/F2.expansion.html>

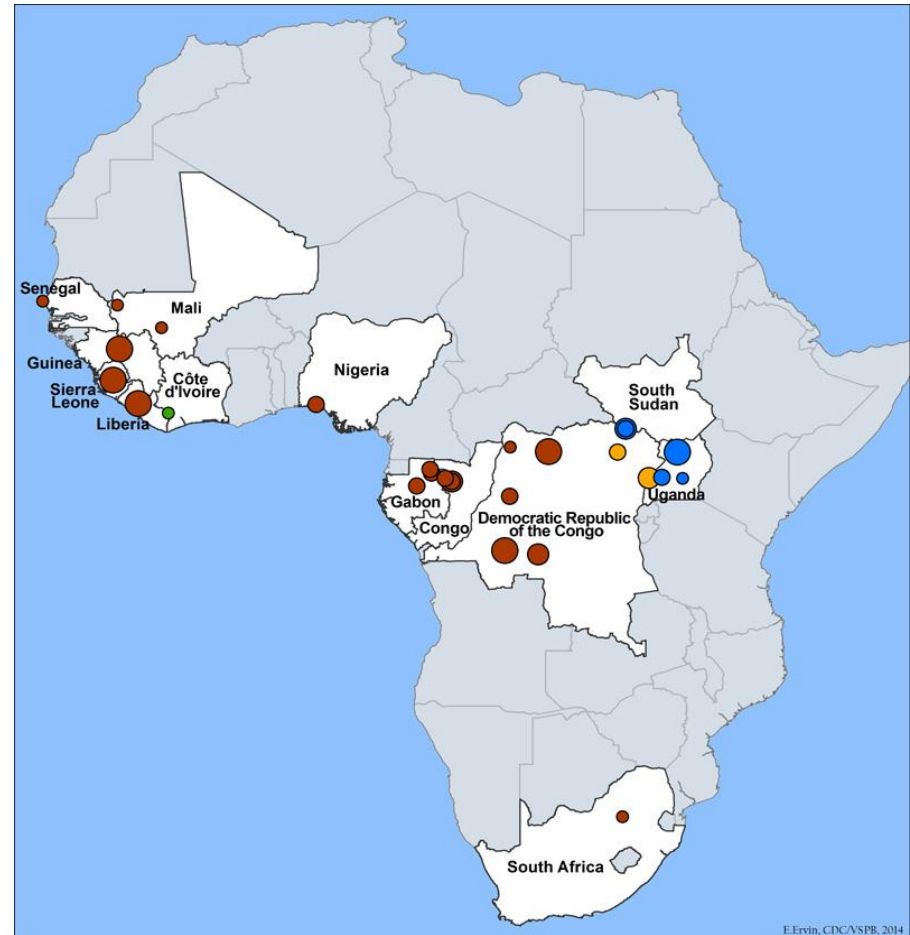


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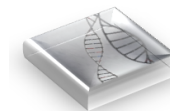
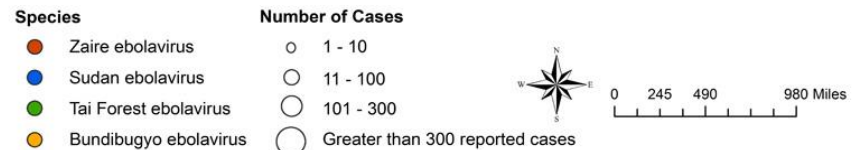
Ebola Makona

SL3864

- One of the first clinical isolates from the recent Ebola outbreak
- Most common lineage during outbreak



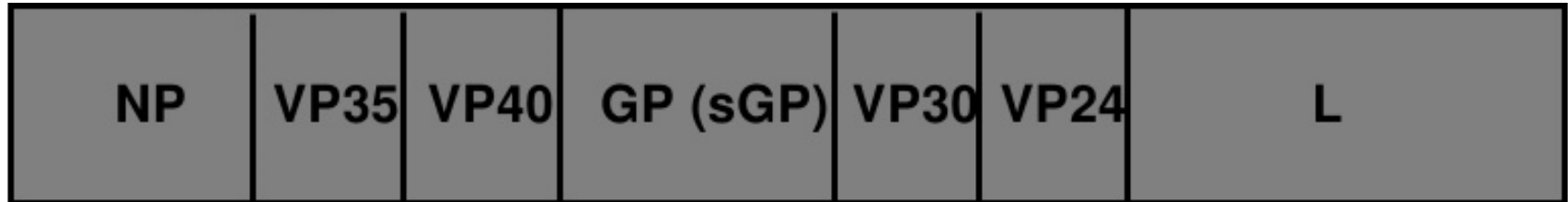
EBOLAVIRUS OUTBREAKS BY SPECIES AND SIZE, 1976 - 2014



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<http://www.cdc.gov>

Synthesize 13 Fragments of SL3864



3. Ligate into complete genome
2. Synthesize 13 pieces, all ~1.5 kb

After each short length ligation, sequence products.

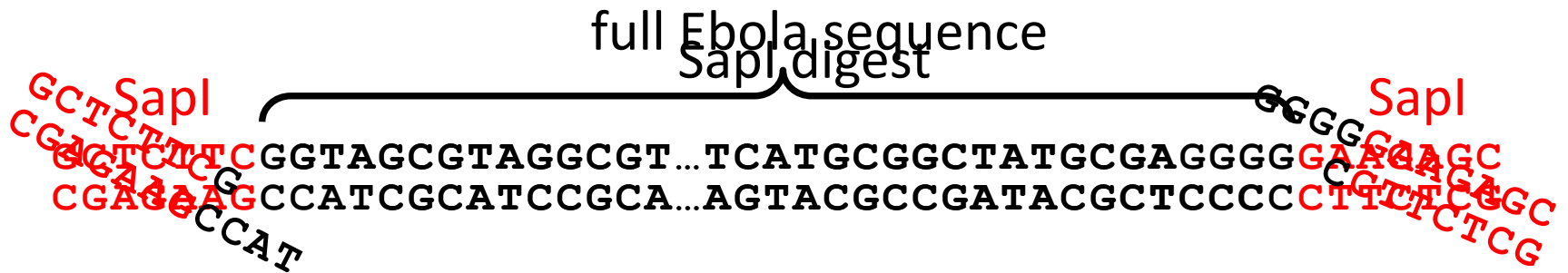
Sanger Sequencing



ABI 3730

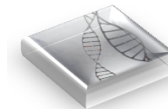
Clone full length SL3864

- ✓ Verified sequences of *short* ligated fragments



Prepare Samples for PacBio RSII

1. Purified plasmid DNA (ZymoPURE™ Plasmid Maxiprep Kit)
2. Fragmented ~21kb plasmids to ~3kb using Covaris LE220
3. 750ng of sheared DNA used for PacBio 2kb Template Prep Procedures
4. Quantified using Qubit and checked size distribution with Agilent 2200 TapeStation (Genomic Kit)



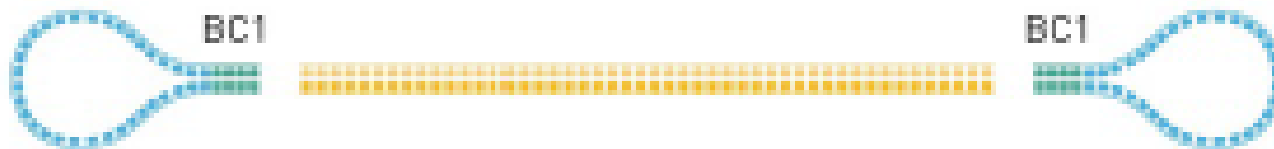
Symmetrical Barcoded Adapters

5. Barcoded adapters allow for multiplexing using a single SMRT Cell

Barcoded End

- 1 pTATGCTAatctctctcttttctcctcctccgttgttggttgagagagatTAGCATA
- 2 pGACAGTGatctctctcttttctcctcctccgttgttggttgagagagatCACTGTC

Barcoded End



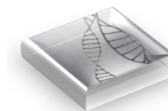
www.pacb.com

Non-Barcoded Adapters

It is not impossible to multiplex in 1 SMRT Cell using non-barcoded adapters.

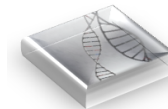
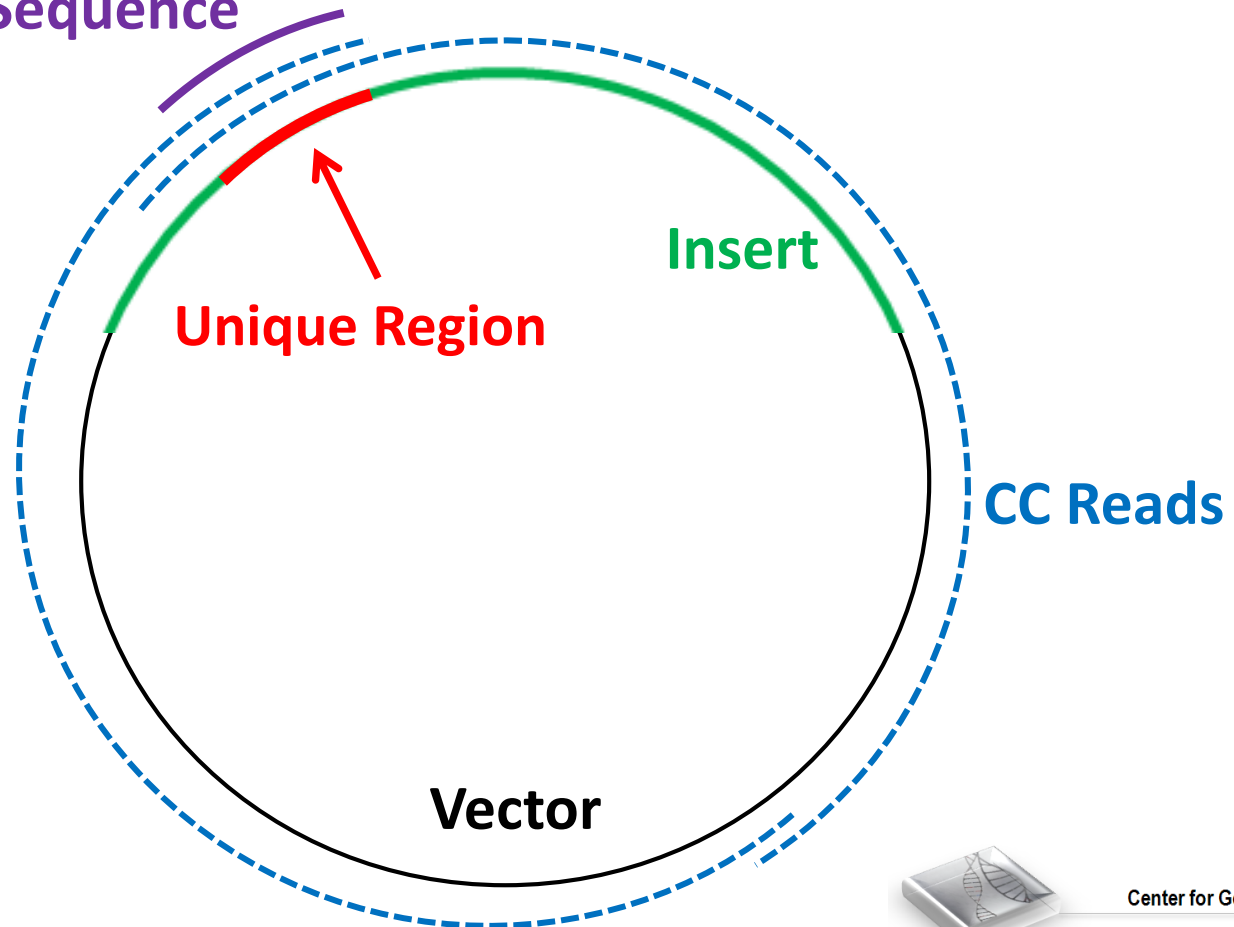
Caveats:

- * Already have an idea about what you are sequencing
- * Each plasmid in the SMRT cell must have a unique sequence region that distinguishes it from the others



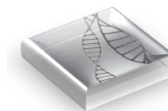
Non-Barcoded Adapters Continued

Reference Sequence



Sequencing With PacBio RSII

6. After two samples were pooled (and barcoded!) they were sequenced in a single SMRT Cell
7. Run Parameters: Average Size = 3kb
 Loading Concentration = 50pM
 4 hour movie

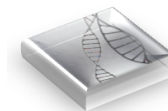


Sequencing With PacBio RSII

| Sample Name | Template Prep Kit | Binding Kit |
|------------------------|-----------------------------------|----------------------------------|
| Makona 2 and 3 mutants | SMRTbell HT Template Prep Kit 1.0 | DNA/Polymerase Binding Kit P6 v2 |

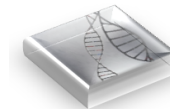
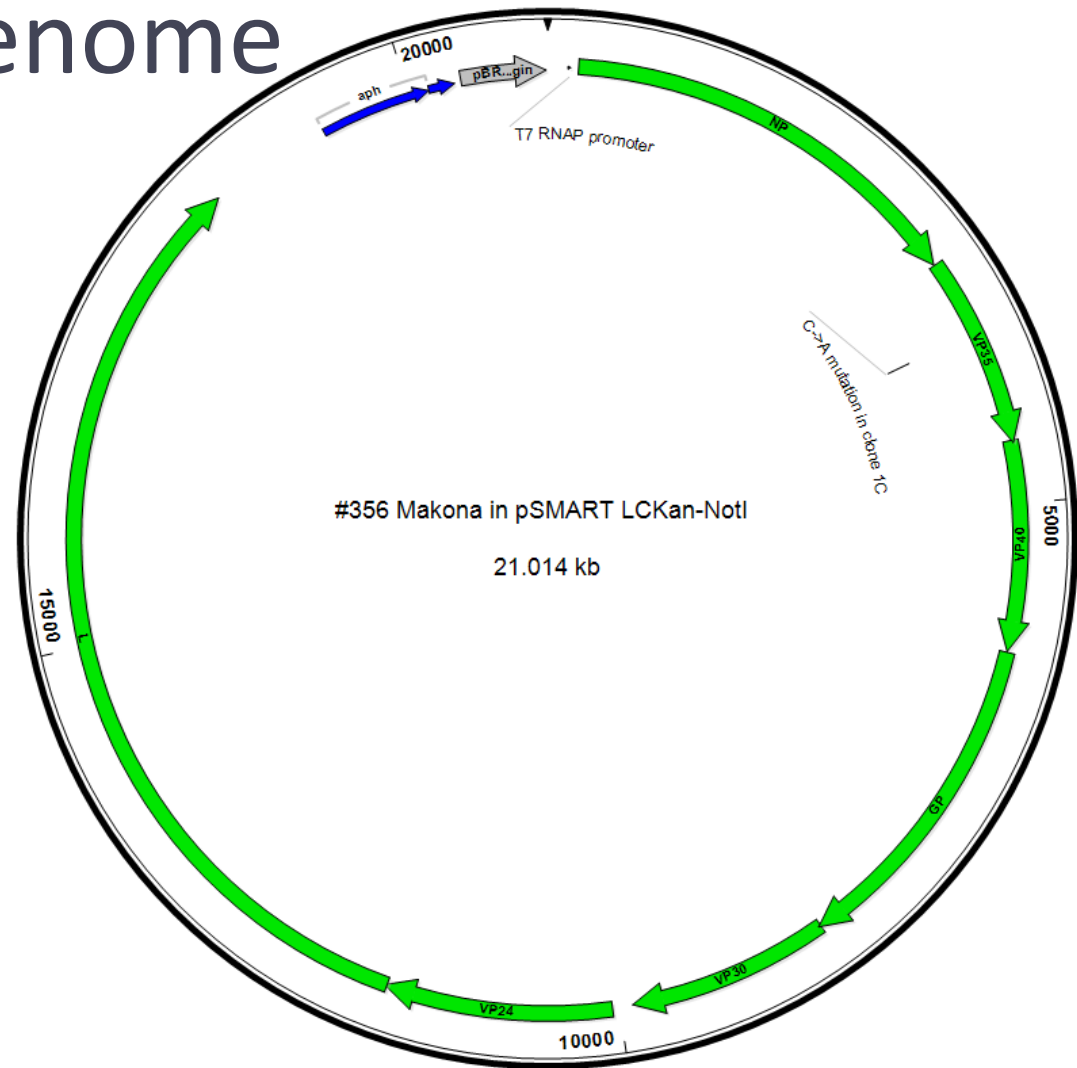
| Collection Protocol | Insert Size (bases) | Stage Start | SMRT Cells | Total Reads | Polymerase Reads | | | Reads Of Insert | | |
|-------------------------|---------------------|-------------|------------|-------------|------------------|---------|--------|-----------------|---------|--------|
| | | | | | Length | Quality | Mbases | Length | Quality | Mbases |
| MagBead Standard Seq v2 | 2000 | Yes | 1 | 65357 | 14940 | 0.85 | 976.5 | 2773 | 0.93 | 181.3 |

| Template | | Productivity | | | Local Base Rate | SNR | |
|---------------|--------------|--------------|-----------------|-------------|-----------------|-----|-----|
| Adapter Dimer | Short Insert | Empty (P0) | Productive (P1) | Other (P2) | | T | A |
| 0.01 | 0.02 | 51458 (34%) | 65357 (43%) | 33477 (22%) | 2.87 | 9.3 | 8.8 |

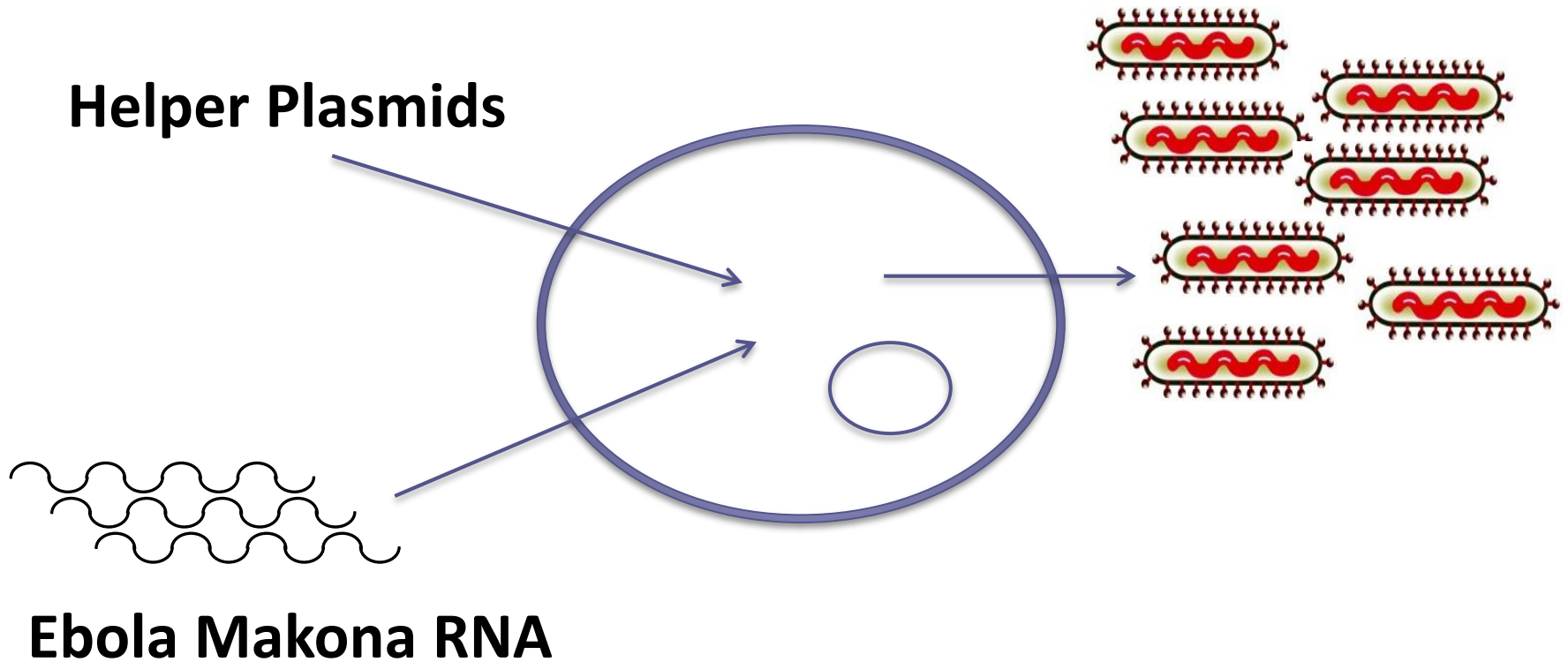


Full Makona Genome

- Successfully cloned SL3864
- The only mutation present is a silent mutation in VP35 (C->A)
- Tag for recombinant virus versus natural isolate



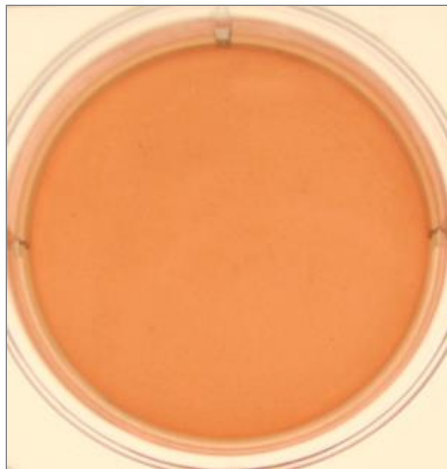
Virus Rescue



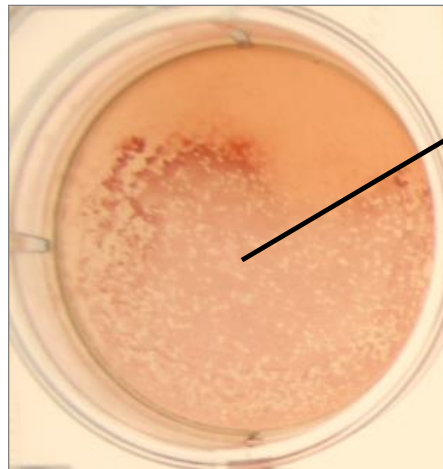
Plaque Assay

- Evidence that virus is present

Negative Control



Experimental



Acknowledgements

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Brett Beitzel

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Galina Koroleva

Thank you.

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