Bring Sanger sequencing to the site of Ebola outbreak

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Ebola virus disease case definition for reporting in EU

http://ecdc.europa.eu/en/healthtopics/ebola_marburg_fevers/EVDcasedefinition/Pages/default.aspx

Clinical criteria

Laboratory criteria

Any of the following:

- Detection of Ebola virus nucleic acid in a clinical specimen and confirmation by sequencing or a second assay on different genomic targets.
- Isolation of Ebola virus from a clinical specimen.

Epidemiological criteria

There are two components of an Ebola virus nucleic acid diagnostic test:

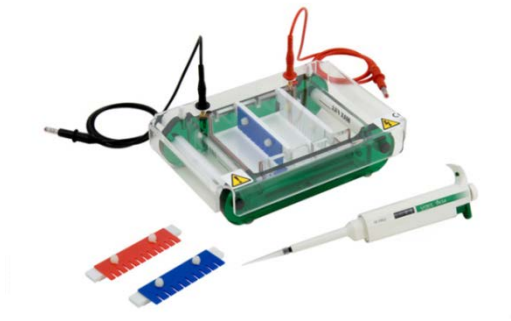
(1) Detection;

(2) Confirmation by DNA sequencing.

Is it Ebola? Malaria? Or MERS?

Perform a same-nested PCR at the site of outbreak in the shade under a tree, then the answer may become obvious.

Equipment needed to perform nested RT-PCR screen and prepare amplicons to be used as sequencing template is inexpensive

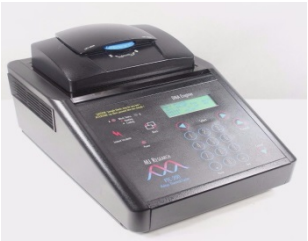


Gel electrophoresis (battery-run)

Primary PCR by pipetting

Nested PCR by microglass rod

UV-viewer (battery-run)



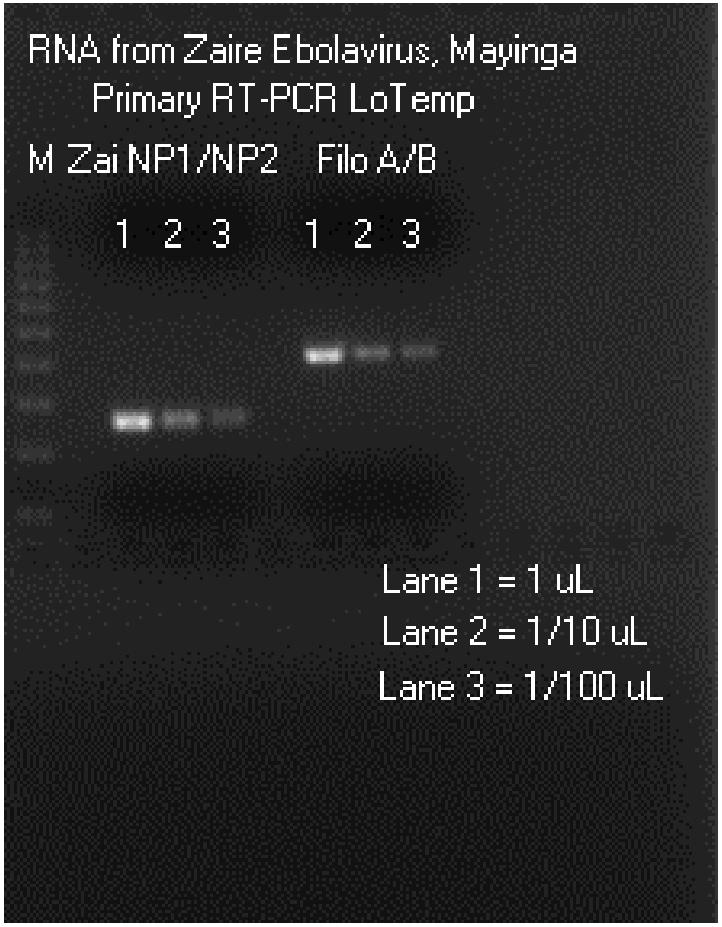
PCR thermal cycler

Small generator for thermal cycler

Detection of RNA from Zaire Ebolavirus Mayinga by LoTemp RT-PCR with 2 standard conserved primers, the Zai NP1/NP2 (nucleoprotein) and Filo A/B (L gene)

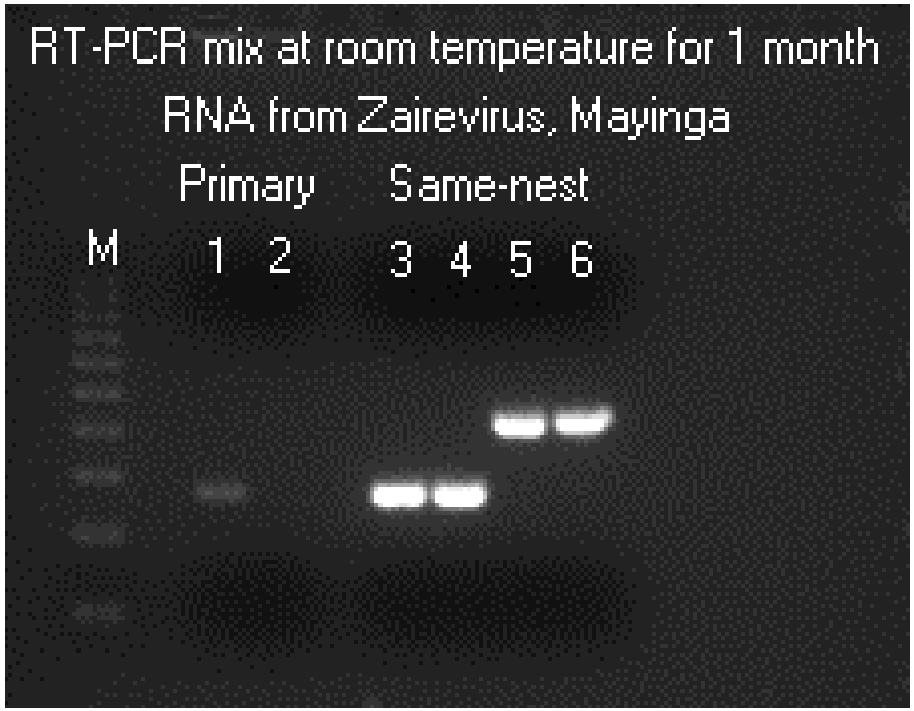
using 10^4, 10^3 and 10^2 copies of viral RNA to initiate each RT-PCR (Lanes 1, 2, 3).

The LoTemp RT-PCR mix containing DNA polymerase, reverse transcriptase, dNTPs, DTT, and ribonuclease inhibitor had been stored **at ambient temperature for 4 weeks, including 3 days at 40°C**



Same-nested PCR can detect about 10 copies of RNA from Zaire Ebolavirus

(Material generously supplied by BEI Resources www.beiresources.org)



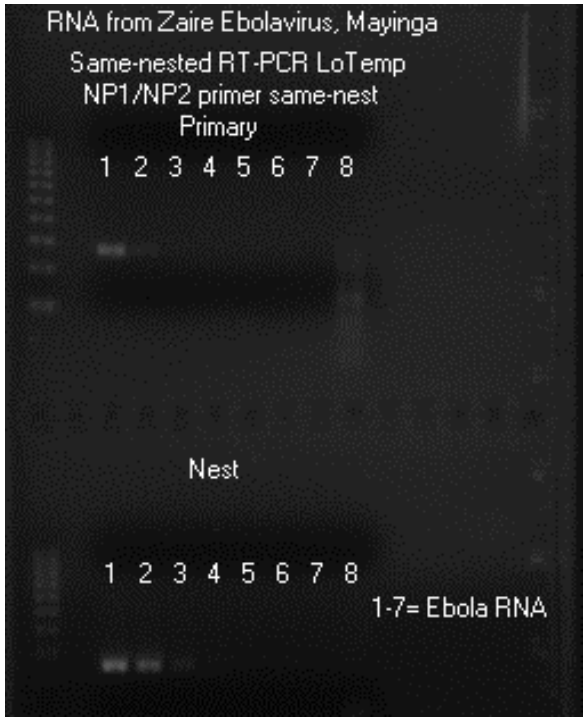
Lanes 1, 3, 4 = 268 bp Zai NP1/NP2 amplicon; Lanes 2, 5, 6 = 419 bp Filo A/B amplicon

Primary PCR condition: 22 µL of LoTemp RT-PCR mix, 1 µL forward primer, 1 µL reverse primer and 1 µL RNA. Nested PCR condition: 20 µL of LoTemp PCR mix, 1 µL forward primer, 1 µL reverse primer and 3 µL water; the nested PCR was initiated by a trace of the primary PCR products transferred by a microglass rod without micropipetting to reduce the chance of aerosol contamination. 30 cycles for primary PCR and 30 cycles for same-nested PCR.

Same-nested PCR increases the detection sensitivity for RNA from Zaire Ebolavirus, Mayinga (Material generously supplied by BEI Resources www.beiresources.org)

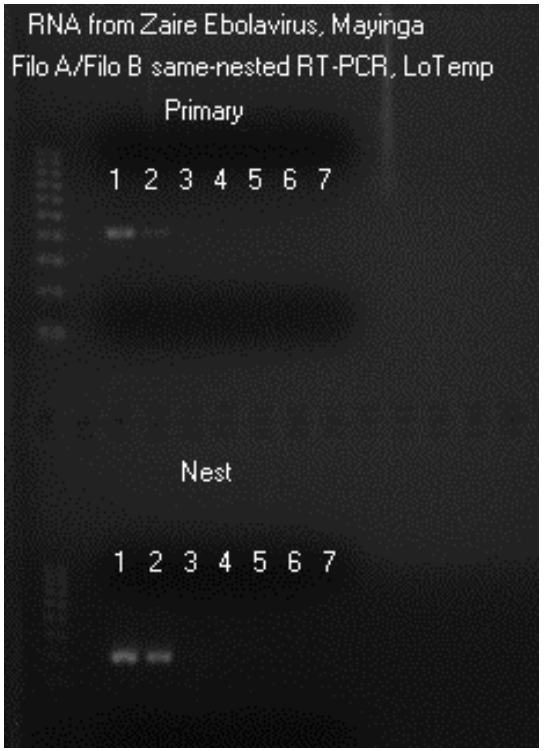
BEI Resources Cat. No. NR-31806, Lot No. 60428456 Ebola RNA diluted in TE buffer to threshold detection level for lane 1 primary PCR, and further 10-fold serially diluted to form a decreasing ladder of concentrations for PCRs in lanes 2-7. Lane 8 was MS2 RNA positive control (band cut off)

Result: Same-nested PCR increases the NP1 and NP2 PCR detection sensitivity by 10-fold over a single PCR



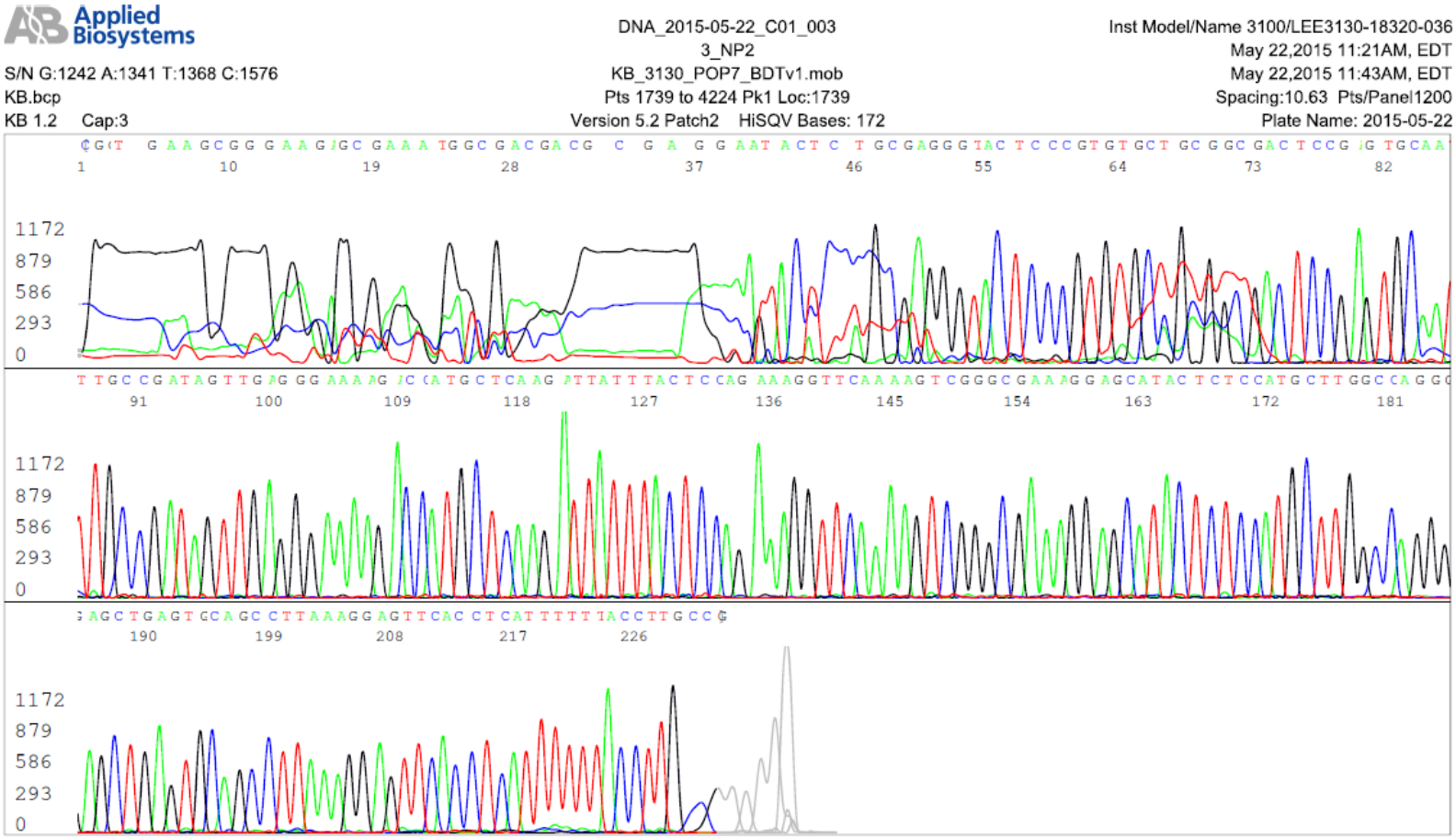
BEI Resources Cat. No. NR-31806, Lot No. 60428456 Ebola RNA diluted in TE buffer to threshold detection level for lane 1 primary PCR, and further 10-fold serially diluted to form a decreasing ladder of concentrations for PCRs in lanes 2-7.

Result= Same-nested PCR increases the Filo A and Filo B PCR detection sensitivity by 10-fold over a single PCR



The non-infectious nested PCR amplicons can be safely transported to a regional laboratory for Sanger sequencing

Computer-generated base-calling electropherogram of a sequence of the Zai NP1/NP2-amplified 268 bp same-nested PCR product-using Zai NP2 as the sequencing primer



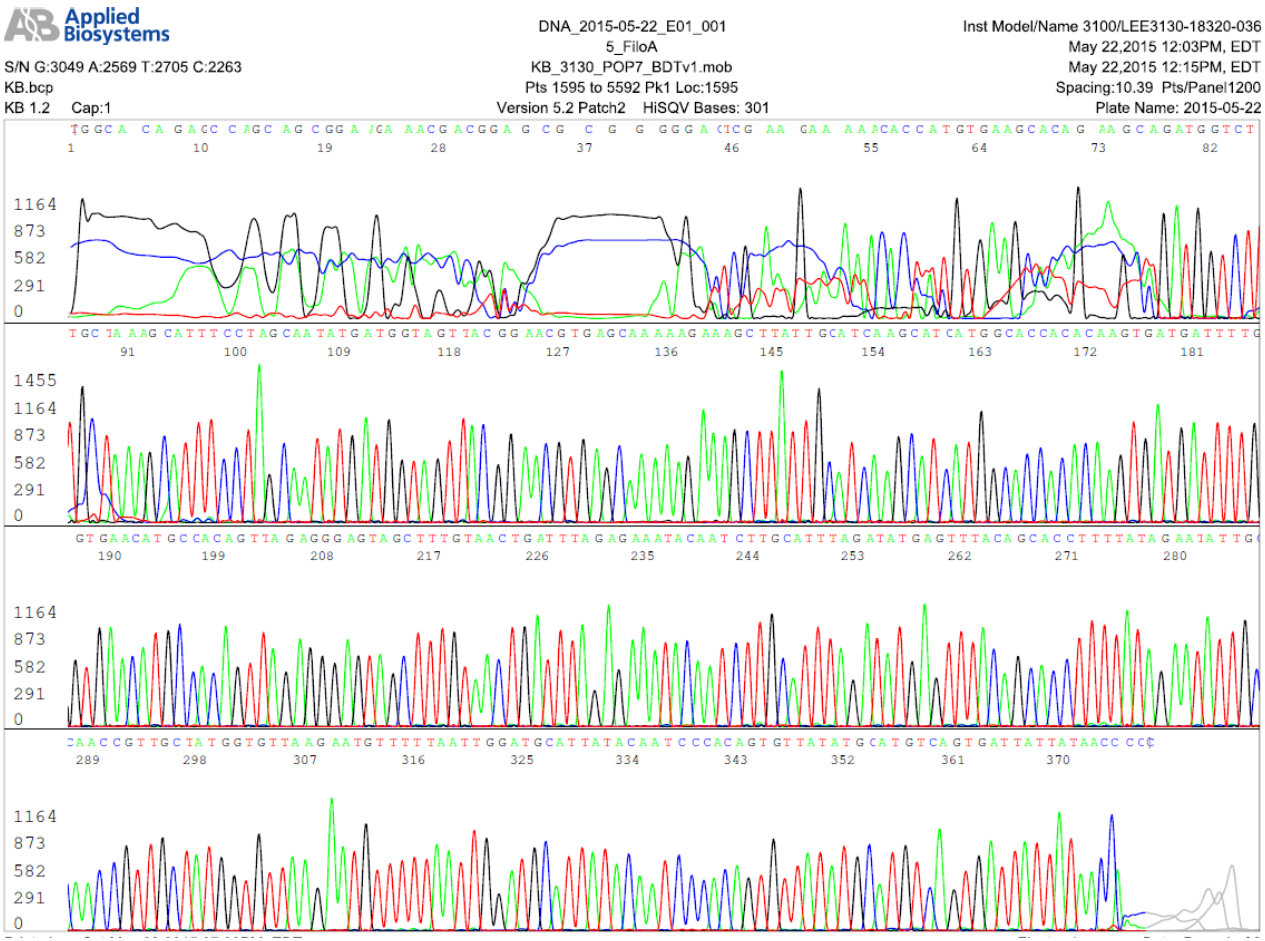
Submission of a 148-base sequence to the GenBank for BLAST alignment confirmed the 268 bp amplicon to be that of RNA from Ebola virus, Mayinga

```
Zaire ebolavirus strain ZEBOV/Homo sapiens-tc/COD/Mayinga_57935/1976, complete genome
Sequence ID: gb|KR063671.1|Length: 18957Number of Matches: 1
Related Information
Range 1: 1281 to 1428GenBankGraphics Next Match Previous Match First Match
            Alignment statistics for match #1
    Score     Expect  Identities     Gaps     Strand   Frame
274 bits(148) 3e-70  148/148(100%) 0/148(0%) Plus/Minus
Features:
Query  1     TGCAATTGCCGATAGTTGAGGGAAAAGACCATGCTCAAGATTATTTACTCCAGAAAGGTT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1428  TGCAATTGCCGATAGTTGAGGGAAAAGACCATGCTCAAGATTATTTACTCCAGAAAGGTT  1369

Query  61    CAAAAGTCGGGCGAAAGGAGCATACTCTCCATGCTTGGCCAGGGAGCTGAGTGCAGCCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1368  CAAAAGTCGGGCGAAAGGAGCATACTCTCCATGCTTGGCCAGGGAGCTGAGTGCAGCCTT  1309

Query  121   AAAGGAGTTCACCTCATTTTTTACCTTG  148
             ||||||||||||||||||||||||||||
Sbjct  1308  AAAGGAGTTCACCTCATTTTTTACCTTG  1281
```

Computer-generated base-calling electropherogram of a sequence of the FiloA/B-amplified 419 bp same-nested PCR product-using Filo A as the sequencing primer



Submission of a 295-base sequence to the GenBank for BLAST alignment confirmed the 419 bp amplicon to be that of RNA from Ebola virus, Mayinga

```
Zaire ebolavirus strain ZEBOV/Homo sapiens-tc/COD/Mayinga_57935/1976, complete genome
Sequence ID: gb|KR063671.1|Length: 18957Number of Matches: 1
Related Information
Range 1: 13326 to 13620GenBankGraphics Next Match Previous Match First Match
            Alignment statistics for match #1
    Score     Expect   Identities     Gaps     Strand  Frame
545 bits(295) 1e-151  295/295(100%) 0/295(0%) Plus/Plus
Features:
Query  1      GATGGTCTTGCTAAAGCATTTCCTAGCAATATGATGGTAGTTACGGAACGTGAGCAAAAA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13326  GATGGTCTTGCTAAAGCATTTCCTAGCAATATGATGGTAGTTACGGAACGTGAGCAAAAA  13385

Query  61     GAAAGCTTATTGCATCAAGCATCATGGCACCACACAAGTGATGATTTTGGTGAACATGCC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13386  GAAAGCTTATTGCATCAAGCATCATGGCACCACACAAGTGATGATTTTGGTGAACATGCC  13445

Query  121    ACAGTTAGAGGGAGTAGCTTTGTAACTGATTTAGAGAAATACAATCTTGCATTTAGATAT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13446  ACAGTTAGAGGGAGTAGCTTTGTAACTGATTTAGAGAAATACAATCTTGCATTTAGATAT  13505

Query  181    GAGTTTACAGCACCTTTTATAGAATATTGCAACCGTTGCTATGGTGTTAAGAATGTTTTT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13506  GAGTTTACAGCACCTTTTATAGAATATTGCAACCGTTGCTATGGTGTTAAGAATGTTTTT  13565

Query  241    AATTGGATGCATTATACAATCCCACAGTGTTATATGCATGTCAGTGATTATTATA  295
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13566  AATTGGATGCATTATACAATCCCACAGTGTTATATGCATGTCAGTGATTATTATA  13620
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A novel low temperature PCR makes it possible

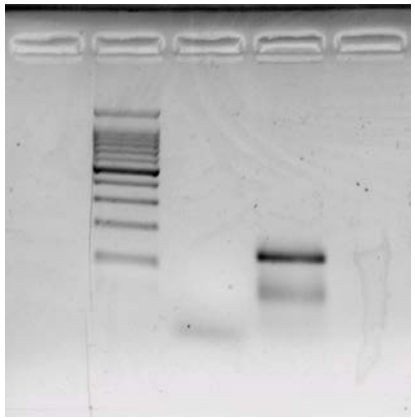
***Low temperature PCR - the gateway to diagnostic Sanger sequencing for infectious diseases: minimize *Taq* errors**

- **A HiFi polymerase(s) for 60-120 cycles of precision PCR amplification**
- **Chemical-assisted denaturing at 85°C, instead of 94-95°C**
- **Chemical stabilizers for enzymes (polymerases, ribonuclease inhibitor and reverse transcriptase) and dNTPs**
- **Melting chemicals to reduce mispriming**
- **Wide-ranged PCR master mix stored at 4-40°C for clinical diagnostics**
- **No pipet transferring of post-PCR products to reduce contamination**
- **No post-PCR purifications before sequencing**
- **Use crude sample for primary PCR**
- **Adjust annealing temperature (40-50°C) for PCR stringency**

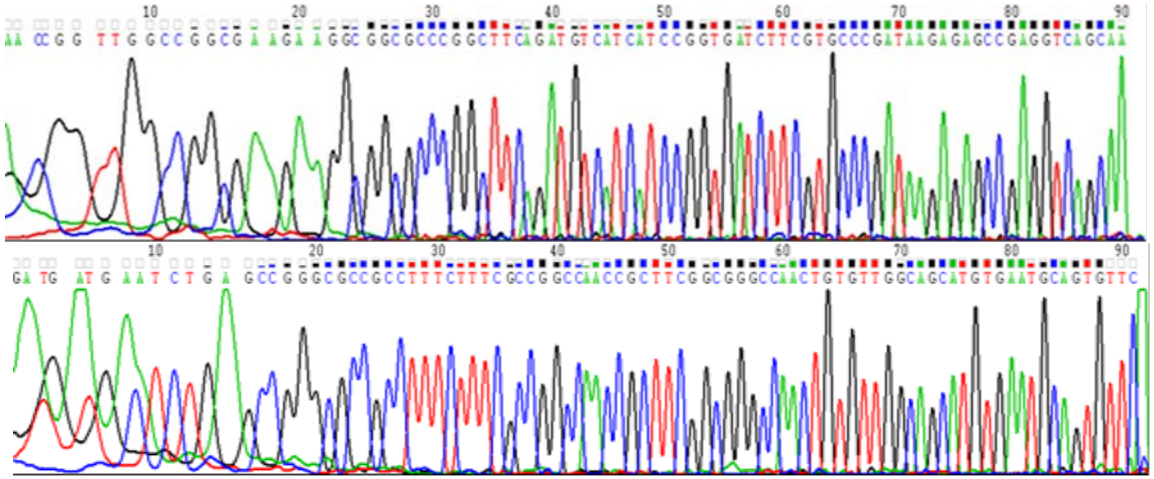
* ***Hong G, Lee SH, Ge S, Zhou S. A Novel Low Temperature PCR Assured High-Fidelity DNA Amplification. International Journal of Molecular Sciences. 2013; 14:12853-12862.***

sRNA from rhizobia and enterobacteria phage MS 2 RNA amplified by RT-PCR mix after 4 weeks storage at 26°C

M H2O 132bp



MS2 RT-PCR



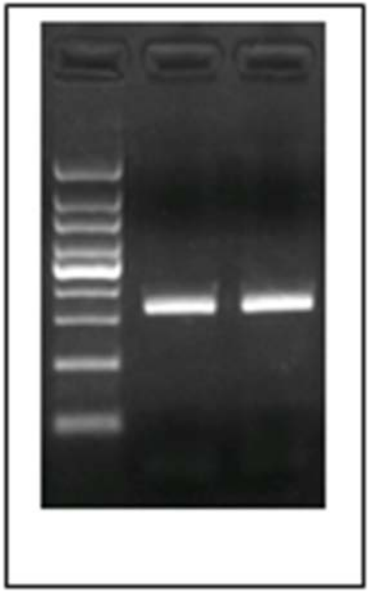
40-cycle low temperature RT-PCR amplification of a grass carp RNA virus causing haemorrhagic disease

Target: 326 bp of viral dsRNA of grass carp reovirus.

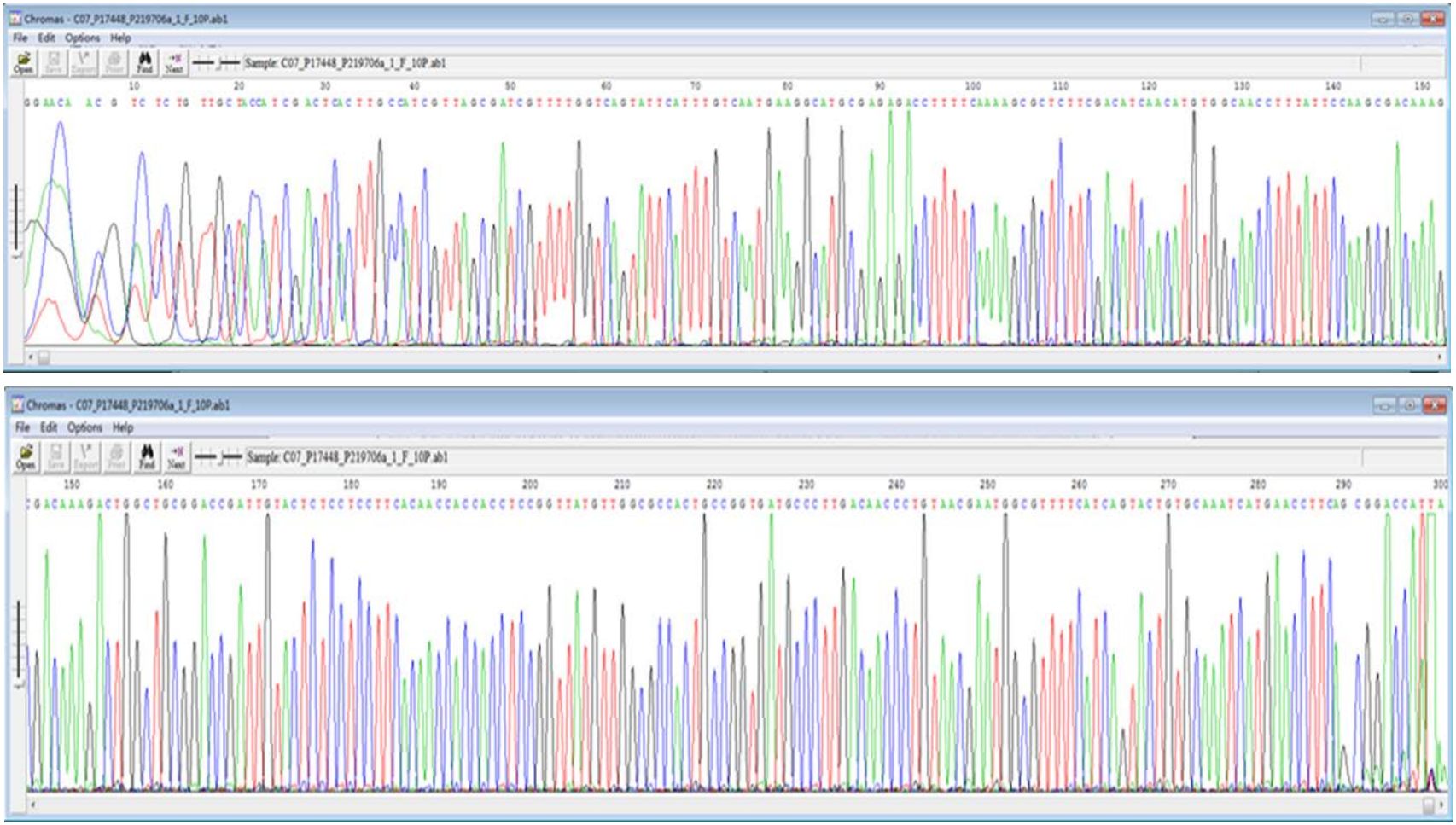
Forward primer:

CTCAATCCTACCGGTAAGCT

Reverse primer:
ATGGTCCGCTGAAGGTTCAT



Diagnostic base-calling electropherogram of the 326 bp of the low temperature RT-PCR amplicon of grass carp reovirus



Appenzeller T. Democratizing the DNA sequence. Science 1990; 247:1030-2.

(PCR was invented to make templates for DNA sequencing, not for diagnosis)

Research News

Democratizing the DNA Sequence

The polymerase chain reaction has made genetic sequences much more easily available, transforming molecular biology and allowing more investigators to work directly with DNA

SCIENCE '90s

Fifth in a series

"I know the term is overused," says William Rainey of the Museum of Vertebrate Zoology at the University of California at Berkeley, "but this is a revolution."

The force driving Rainey's revolution is the polymerase chain reaction, or PCR. Only a couple of years ago, researchers found it extremely laborious to obtain a specific stretch of DNA in quantity from the mass of genes in a biological sample. By making that task easy, PCR is having profound effects on the work of genome mappers, AIDS researchers, evolutionary biologists like Rainey, developmental biologists, medical diagnosticians, and even forensic scientists. In many areas PCR has cut across the boundaries separating basic research and applied, commercial technology in a way few techniques ever have.

PCR has already turned museum collec-

ing of DNA's four nucleotides, its chemical building blocks. Add a DNA polymerase (an enzyme that, given one strand of DNA, assembles the complementary strand). Add two primers (strands of DNA perhaps 20 nucleotides long chosen to be complementary to regions at opposite ends of the target sequence) to start the reaction.

This mixture is heated and cooled in cycles of a few minutes each. The first round of heating separates the double-stranded DNA into two single strands. Then, as the mixture cools, the primers find their complementary sequences, and the DNA polymerase gets to work, extending each primer into a new DNA strand.

That much is straightforward. The trick is that the next cycle of heating separates the copies from the original strands—and *both* sets become templates for a new round of DNA synthesis. As a result of this doubling, the target DNA multiplies exponentially—in a chain reaction. Thirty cycles of PCR—no more than an afternoon's work—can amplify a molecular signal that was too small

shake Cetus's domination of the business of PCR, it certainly will not stop the scientific juggernaut that is already under way. Even Mullis, who has the natural pride of a new father, confesses to being overwhelmed by how quickly his baby caught on. According to a "PCR Bibliography" published by the Perkin-Elmer–Cetus partnership, 476 papers appeared last year describing work in which PCR played a central role.

From this welter of applications a few themes seem to be emerging that will be of increasing significance during the 1990s. One theme is the transformation of the study of evolution by a wave of DNA data. Allan Wilson of the University of California at Berkeley deciphers evolutionary relations by comparing genetic material from different organisms. Since his laboratory began using PCR, he has been swamped, he says: "With the same number of people in the laboratory, the rate of data production has accelerated so I can't keep up with it."

A particularly dramatic example of how PCR has speeded things up is the reanalysis

Sanger sequencing of 16S rRNA gene showing mixed infections of *B. burgdorferi* and one of *B. miyamotoi* in a single patient - three characteristic double base peaks marked Z,Y,X.

Lee SH et al. Inter J Mol Sci. 2014; 15:11364-11386.

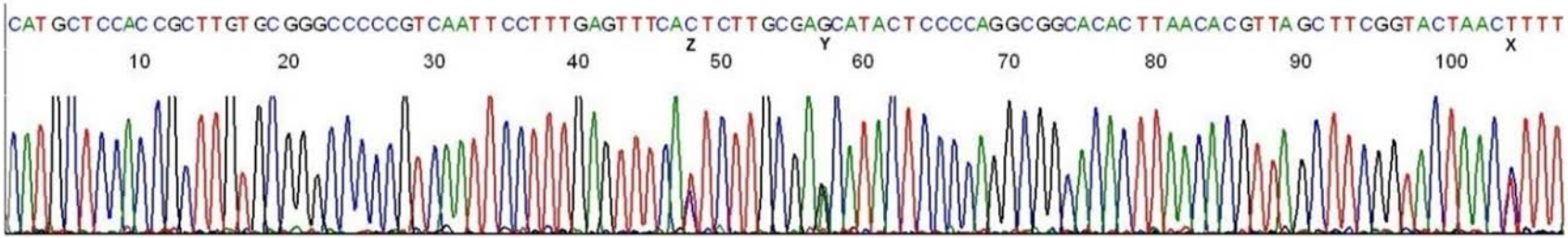
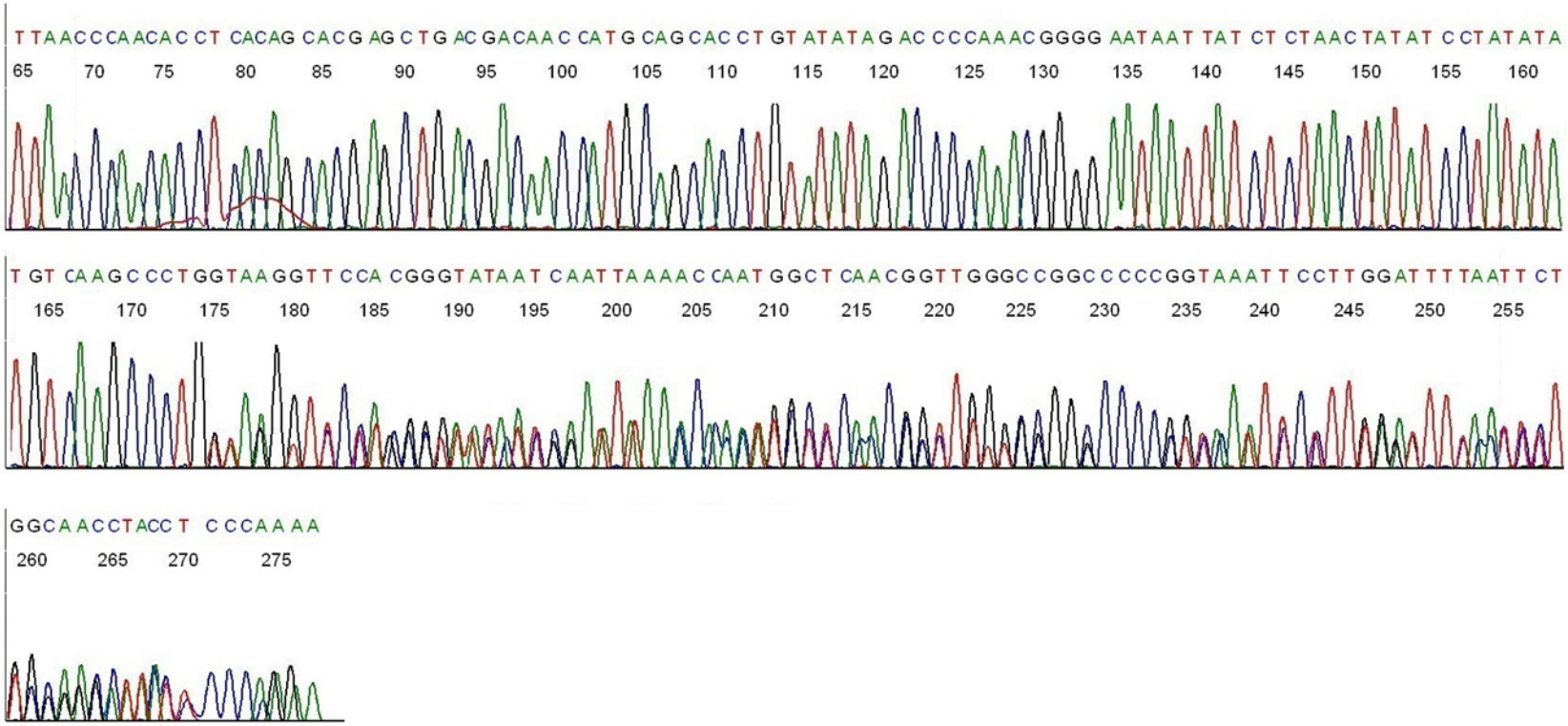


Fig. 8

```
#1 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAGCATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACTTTT
#2 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACCTTT
#3 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACTTTT
#4 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAGCATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACCTTT
#5 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAGCATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACTTTT
#6 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAACATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACCTTT
#7 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAACATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACTTTT
#8 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAGCATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACCTTT
```

Wild *Borrelia burgdorferi* isolate from a patient's blood with more than 1 copy of 16S rRNA gene

Case 2014 02 21 (template prepared by same-nested PCR after 60-cycle amplification)
To be presented at the AACC July 2015 meeting



Lower the PCR temperatures by 10°C to prepare Sanger sequencing template

You can detect infectious agents for Lyme disease, Ebola, Malaria, Cholera, Mers…at site of outbreak with

- **Extremely high specificity**
- **Extremely high sensitivity**
- **Very practical**
- **Financially sustainable**

Bring the LoTemp RT-PCR and PCR reagents in your carry-on to the field lab in West Africa (No freezing, no dry ice)