Revolutionizing Multiplex PCR: A Proof-of-Concept Study Demonstrating Four-Target Detection (Influenza A and B, RSV, and IC) with Two Fluorescence Channels in One Tube

Amplidea

RES-296

JungKwon Yoon[1], Jaehyeon Lee[2]

[1] Amplidea, Jeonju, Republic of Korea

[2] Department of Laboratory Medicine, Jeonbuk National University Medical School and Hospital, Jeonju, Republic of Korea

Backgrounds

- Multiplex real-time PCR is a cornerstone of respiratory-virus diagnostics.
- Limited number of fluorescence channels constrains simultaneous target detection.
- **FluoroSplit** technology: signal-splitting platform that resolves two targets within a single channel (figure 1)
  - On a five-channel real-time PCR platform, up to 10 distinct targets can be simultaneously detected using the FluoroSplit
  - Expands multiplex capacity without additional hardware.
- This is a Proof-of-concept study
  - four-target assay detecting Influenza A (FluA), Influenza B (FluB), RSV, and an internal control (IC) in one tube.
  - Presenting proof-of-concept data and a preliminary clinical evaluation of prototype reagent.

Methods

- A four-target multiplex assay using FluoroSplit was developed.
  - Platform: CFX96 Touch instrument (Bio-Rad, USA).
  - Channels:
    - FAM → FluA and FluB
    - Cy5 → RSV and IC
    - Acquisition: dual fluorescence acquisition at 62 ˚C and 84 ˚C.
- Specimens: 34 RNA extracts from clinical nasopharyngeal swabs (previously tested by Allplex Respiratory Panel, Seegene, Republic of Korea).
- Contrived mixtures (n=10): to confirm clear target separation within a single channel
  - 2 mixtures with FluA + FluB
  - 4 mixtures with FluB + RSV
  - 4 mixtures with FluA + RSV
- Statistical Analysis: Agreement statistics using linear-weighted kappa calculated with MedCalc 23.3.2.

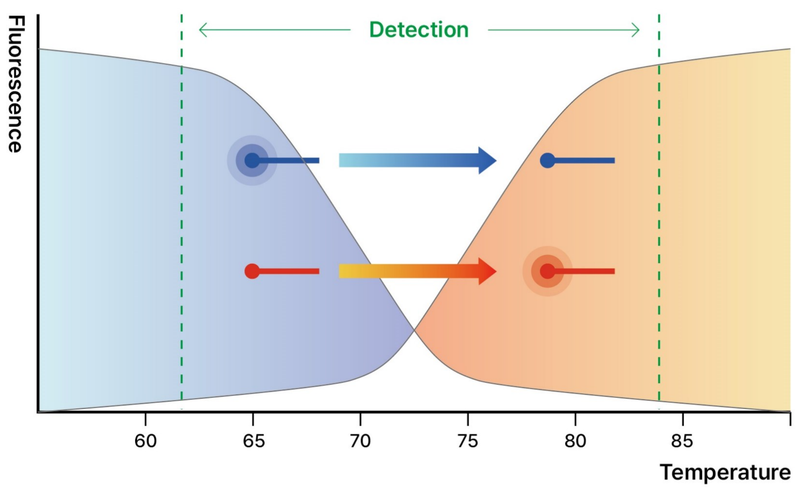

Figure 1. This technology separates fluorescence signals by temperature, enabling each target to be detected independently in real-time PCR and producing separate Ct values. Unlike melting curve analysis, it requires no additional post-run analysis, allowing amplification of each target to be monitored in real time.

Results

- Concordance: Weighted kappa = 0.81 (95 % CI, 0.64-0.95).
  - Missed detections: 5 high-Ct specimens (3 FluA, 2 RSV).
- Contrived samples: 90 % differentiation achieved.
  - FluA and FluB separated in FAM.
  - RSV and IC separated in Cy5.
  - Internal control: Recovered in every reaction, confirming assay integrity.

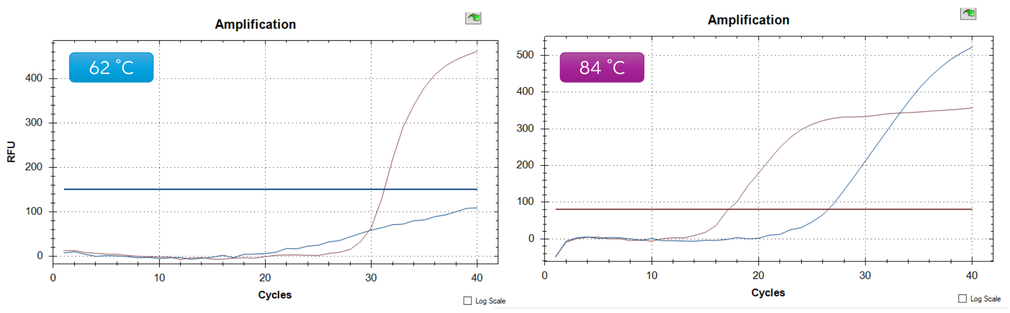

Figure 3. Contrived mixture: FluA + RSV. The internal control amplifies in Quasar 670 at 62 ˚C (left). At 84 ˚C, distinct curves for FluA (FAM) and RSV (Quasar 670) are visible in the right.

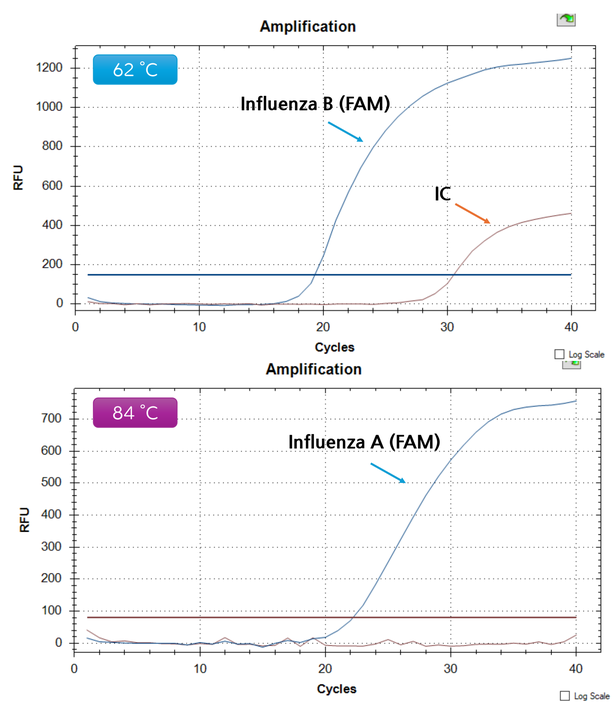

Figure 2. Contrived mixture: FluB + FluA. In the left, FluB is observed in FAM at 62 ˚C and the IC in Quasar 670. The right figure shows the Influenza A(FluA) curve in FAM at 84 ˚C. Note how the FluB signal at 62 ˚C and the FluA signal at 84 ˚C remain cleanly separated without interference.

Conclusion

- FluoroSplit assay enables accurate detection of four respiratory targets using only two fluorescence channels.
- **Proof-of-concept**: <u>two targets can be distinctly resolved within a single channel</u>. On five-channel platforms (e.g., CFX96), approach could support decaplex assays.
- Operates via temperature-dependent signal acquisition only:
  - No specialized hardware required.
  - No post-run mathematical deconvolution required.
  - Can be implemented immediately on standard platforms (e.g., CFX96).
- Particularly advantageous for: 1) Resource-limited settings, 2) Syndromic panels constrained by instrument capability.
- Findings underscore potential of FluoroSplit to deliver high-efficiency molecular diagnostics with minimal fluorescence bandwidth requirements for clinical laboratory practice.

Contact information 1
JungKwon Yoon
Email: CEO@amplidea.co.kr

Contact information 2
Jaehyeon Lee, M.D., Ph.D.
Email:JLEE@jbnu.ac.kr, Laboratorymedicine@gmail.com

APCCMI2025 BANGKOK