

IMPERIAL

Optimising Multiplex Molecular Diagnostics for Infectious Diseases Through Advanced Signal Processing and Machine Learning

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Statement of Originality

I hereby declare that this dissertation and the work described in it are my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, except where due acknowledgement has been made in the text.

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Ke Xu

Abstract

Rapid, low-cost Nucleic Acid Amplification Tests (NAAT) are essential for the diagnosis of infectious diseases. However, existing multiplexing polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) tests struggle with reliability and cost. Amplification Curve Analysis (ACA) offers a data-driven alternative for single-tube multiplex tests, but kinetic factors hinder its reliability. Addressing these data and instrumentation challenges motivates the data-driven multiplexing framework advanced in this thesis.

In addition, colorimetric LAMP (cLAMP) offers an inexpensive, equipment-light choice for point-of-care NAAT. However, fully automating its readout remains difficult, highlighting the need for a universal, hardware-independent colour-detection method, which serves the other aim of the thesis.

This thesis charts data-driven approaches to multiplexing pathways from amplification curves to colorimetric tests for the detection of infectious diseases, integrating signal processing, machine learning and biochemical design. Chapter 1 familiarises readers with background information and elaborates on the challenges for data-driven implementation in NAAT. Chapter 2 presents an Adaptive Mapping Filter that removes non-specific and low-efficiency digital PCR curves, providing a standard data processing procedure. Chapter 3 delivers Smart-Plexer 2.0, using robust kinetic features and clustering to automate single-well multiplex PCR assay selection. Chapter 4 tackles the domain shift of PCR data with a transformer-based adversarial network, enabling label-free adaptation. Chapter 5 automates cLAMP by pairing deep image segmentation with adaptive colour identification, achieving 99% accuracy on clinical samples. Chapter 6 re-engineers LAMP kinetics with a primer-ratio optimisation, generating distinct Time-to-Positive for separate targets, expanding single-channel multiplexing in real-time LAMP.

In conclusion, this thesis advances multiplex diagnostics from biochemical and informatics perspectives. It demonstrates the integration of adaptive filtering, machine-driven assay selection, domain-robust learning, and machine learning-based automation for improving diagnostic precision and accessibility, providing a blueprint for next-generation assays that are not only more accurate but also smarter—capable of learning from every output they generate.

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List of Abbreviations

AC: Amplification Curve

ACA: Amplification Curve Analysis

Adam: Adaptive Moment Estimation

ADS: Average Distance Score

A/H1N1: influenza A virus H1N1

A/H1N1/09: influenza A virus H1N1 2009 or swine flu

A/H3H2: influenza A virus H3N2

AMCA: Amplification and Melting Curve Analysis

AMF: Adaptive Mapping Filter

AMR: Antimicrobial Resistance

AUC: Area Under Curve

Bi-LSTM: Bidirectional Long Short-Term Memory Network

Bst: *Bacillus stearothermophilus*

CCA: Connected Component Analysis

CDAN: Conditional Domain Adversarial Network

cDNA: Complementary DNA

cGAN: Conditional Generative Adversarial Network

cLAMP: Colorimetric Loop-Mediated Isothermal Amplification

CNN: Convolutional Neural Network

DA: Domain Adaptation

DBSCAN: Density-based Spatial Clustering of Applications with Noise

DDM: Data-Driven Multiplexing

ddPCR: Droplet dPCR

DNA: Deoxyribonucleic Acid

dPCR: Digital PCR

dsDNA: Double-Stranded DNA

FFI: Final Fluorescence Intensity

FRET: Förster Resonance-Energy Transfer

GRL: Gradient Reversal Layer

HAdV: human adenovirus

HCoV-229E: human coronavirus 229E

HCoV-HKU1: human coronavirus HKU1
HCoV-NL63: human coronavirus NL63
HCoV-OC43: human coronavirus OC43
HDPCR: High-Definition PCR
hIAV or FluA: influenza A virus
hIBV or FluB: influenza B virus
HRV: human rhinovirus
HSV: human herpesvirus
IFC: Integrated Fluidic Circuit
i.i.d.: Independent and Identically Distributed
ILI: Influenza-Like Illness
KNN: k-Nearest Neighbor
LAMP: Loop-Mediated Isothermal Amplification
LFT: Lateral Flow Test
LN: Layer Normalisation
LOCO: Leave-One-Concentration-Out
LOD: Limit of Detection
LOF: Local Outlier Factor
LS: Least-Squares
MC: Melting Curve
MCA: Melting Curve Analysis
MDS: Minimum Distance Score
MERS-CoV: Middle East respiratory syndrome-related coronavirus
ML: Machine Learning
MLP: Multilayer Perceptron
MMD: Maximum Mean Discrepancy
mNGS: Metagenomic Next-generation Sequencing
mpox: monkeypox virus
MSA: Multi-Head Self-Attention
MSC: Multidimensional Standard Curve
MSE: Mean Squared Error
MSS: Mean Silhouette Score
NAAT: Nucleic Acid Amplification Test
OC-SVM: One-Class Support Vector Machine

OPXV: orthopoxvirus
PCA: Principal Component Analysis
PCR: Polymerase Chain Reaction
Pf: *Plasmodium falciparum*
PI: Percentile Interval
Pk: *Plasmodium knowlesi*
Pm: *Plasmodium malariae*
POC: Point of Care
Pow: *Plasmodium ovale wallikeri*
PSO: Particle Swarm Optimisation
Pv: *Plasmodium vivax*
qdPCR: Real-Time Quantitative Digital PCR
qPCR: Real-Time Quantitative Polymerase Chain Reaction
RCA: Rolling Circle Amplification
RF: Random Forest
RMSE: Root Mean Squared Error
RNA: Ribonucleic Acid
ROI: Region-of-Interest
ROC: Receiver Operating Characteristics
RSV or hRSV: respiratory syncytial virus
RTI: Respiratory Tract Infection
RT-LAMP: Reverse-Transcription LAMP
RT-PCR: Reverse-Transcription PCR
SADDLE: Simulated Annealing Design using Dimer Likelihood Estimation
SARS-CoV-2: severe acute respiratory syndrome coronavirus 2
Spn: *Streptococcus pneumoniae*
ssDNA: Single-Stranded DNA
STD: Standard Deviation
SVM: Support-Vector Machine
T-CDAN: Transformer-based Conditional Domain Adversarial Network
TRF: Trust Region Reflective
t-SNE: t-Distributed Stochastic Neighbor Embedding
TTP: Time-to-Positive
ULOD: Upper Limit of Detection

List of Abbreviations

VZV: varicella zoster virus

WMP: Wrong Melting Percentage

Peer-Reviewed Journal Articles Directly Related to Thesis Work

1. L. Miglietta*, K. Xu*, P. Chhaya, L. Kreitmann, K. Hill-Cawthorne, F. Bolt, A. Holmes, P. Georgiou, J. Rodriguez-Manzano, ‘Adaptive Filtering Framework to Remove Nonspecific and Low-Efficiency Reactions in Multiplex Digital PCR Based on Sigmoidal Trends’, *Analytical Chemistry*, vol. 94, no. 41, pp. 14159–14168, Oct. 2022. *Joint first authorship
2. Y. Mao*, K. Xu*, L. Miglietta, L. Kreitmann, N. Moser, P. Georgiou, A. Holmes, J. Rodriguez-Manzano, ‘Deep Domain Adaptation Enhances Amplification Curve Analysis for Single-Channel Multiplexing in Real-Time PCR’, *IEEE Journal of Biomedical and Health Informatics*, vol. 27, no. 6, pp. 3093–3103, Jun. 2023. *Joint first authorship
3. K. Xu*, L. Miglietta*, P. Kesakomol, A. Holmes, P. Georgiou, N. Moser, J. Rodriguez-Manzano, ‘Smart-Plexer 2.0: Leveraging New Features of Amplification Curves to Enhance the Selection of Multiplex PCR Assays in Multi-Target Identification’, *Analytical Chemistry*, vol. 97, no. 27, pp. 14311–14320, Jul. 2025. *Joint first authorship
4. L. Kreitmann, L. Miglietta, K. Xu, K. Malpartida-Cardenas, G. D’Souza, M. Kaforou, K. Brengel-Pesce, L. Drazek, ‘Next-generation molecular diagnostics: Leveraging digital technologies to enhance multiplexing in real-time PCR’, *TrAC Trends in Analytical Chemistry*, vol. 160, p. 116963, Mar. 2023.
5. L. Miglietta, Y. Chen, Z. Luo, K. Xu, N. Ding, T. Peng, A. Moniri, L. Kreitmann, M. Cacho-Soblechero, A. Holmes, P. Georgiou, J. Rodriguez-Manzano, ‘Smart-Plexer: a breakthrough workflow for hybrid development of multiplex PCR assays’, *Communications Biology*, vol. 6, no. 1, pp. 1–16, Sep. 2023.

Manuscripts Under Review

1. K. Xu, M. Cavuto, K. Malpartida-Cardenas, M. Pond, D. Robinson, F. Kiemde, B. Hernandez, S. Sriskandan, U. D’Alessandro, A. Erhart, H. Tinto, A. Cunningham, A. Holmes, P. Georgiou, N. Moser, J. Rodriguez-Manzano, ‘AI-Driven Workflow for Automated Colorimetric Isothermal Amplification for Robust Pathogen Detection on Portable Devices’, under review. <http://dx.doi.org/10.2139/ssrn.4975777>

Additional Peer-Reviewed Articles During PhD

1. S. Peng, Y. Li, R. Cui, K. Xu, Y. Wu, M. Huang, C. Dai, T. Tamur, S. Mukhopadhyay, C. Chen, W. Chen, ‘Sleep postures monitoring based on capacitively coupled electrodes and deep recurrent neural networks’, *Biomedical Engineering Online*, vol. 21, no. 1, p. 75, Oct. 2022.
2. Y. Wu, K. Xu, S. Peng, Y. Cao, Y. Huang, R. Cui, H. Zhu, Y. Guo, Z. Wang, C. Dai, W. Chen, ‘Clinical Validation of a Capacitive Electrocardiogram Cushion Utilized for Arrhythmias Monitoring’, *IEEE Transactions on Instrumentation and Measurement*, vol. 72, pp. 1–13, 2023.
3. X. Tan, Y. Huang, K. Xu, D. Zhang, C. Dai, and D. Zhao, ‘Multi-Level Signal Quality Assessment Approach for Multi-Channel Non-Contact Smart Mattress Systems’, *IEEE Sensors Journal*, 2025. <https://doi.org/10.1109/JSEN.2024.3486781>
4. Y. Yuan, Y. Wu, Y. Guo, C. Dai, K. Xu[†], ‘A Real-Time High-Density sEMG Gesture Recognition System Distilled from a Deep Model’, *The Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC) 2025*, accepted (in press).
[†]Corresponding author
5. X. Tan, Y. Zhang, W. Chen, K. Xu[†], C. Dai[†], ‘Oct-HD: A Wearable Distributed Wireless HD-sEMG Synchronous Acquisition System for Long-Term Monitoring’, *IEEE Internet of Things Journal*, vol. 12, no. 15, pp. 32245-32258, Jun 2025. [†]Corresponding author and joint last authorship

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<https://scholar.google.com/citations?user=YMgP0qIAAAAJ>

Chapter 1 Introduction

1.1 Motivation

The imperative for swift, accurate, and comprehensive detection of infectious agents has never been clearer, as recent large-scale outbreaks have demonstrated how rapid diagnostics can reduce both clinical morbidity and societal disruption [1]. Nucleic acid amplification tests (NAAT), most notably the polymerase chain reaction (PCR) and the loop-mediated isothermal amplification (LAMP) chemistries, have emerged as the backbone of modern molecular diagnostics because they combine exquisite analytical sensitivity with rapid turnaround time [2]. Since they amplify targets exponentially, these assays provide answers much faster than culture techniques at a relatively low cost, making them suitable for large-scale screening [2].

The COVID-19 pandemic provided compelling proof of concept: PCR-based workflows enabled high-throughput screening that guided isolation policies, contact tracing, and therapeutic decisions worldwide [3]. Similar benefits extend to routine clinical contexts, where PCR or LAMP panels can resolve complex syndromes, such as influenza-like illness or sepsis, within a single work shift, supporting timely and targeted therapy [4].

When benchmarked against alternative multi-pathogen detection platforms, the advantages of NAAT become even more pronounced. Oligonucleotide microarrays offer broad target coverage but rely on post-amplification hybridisation, which lengthens the workflow, and their analytical sensitivity can be as much as 50% lower compared with real-time PCR [5], [6]. Metagenomic next-generation sequencing (mNGS) offers unparalleled breadth and the capacity to discover novel agents; however, the high consumable costs and sophisticated bioinformatics required hinder its use in broad clinical applications, particularly in resource-limited settings [7]. In contrast, PCR and LAMP maintain near-single-copy limits of detection, require only standard thermocyclers or relatively low-cost heaters, and achieve reasonable reagent costs per reaction in laboratory-developed tests [8], [9]. Collectively, these attributes render nucleic acid amplification the most pragmatic, scalable, and cost-effective strategy for front-line pathogen diagnostics.

While successful, traditional PCR or LAMP applications generally focus on detecting a single pathogen per test, especially in low-resource environments, which restricts throughput and increases the time, costs, and sample volume required for thorough diagnosis [10]. However,

real-world clinical situations often require the simultaneous detection of multiple pathogens, particularly for diseases that share similar clinical symptoms.

For instance, Influenza-like Illnesses (ILIs) consist of various respiratory infections caused by multiple pathogens, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenza viruses, rhinoviruses, and respiratory syncytial virus (RSV) [11]. Accurately identifying these pathogens is crucial for providing effective patient care and implementing appropriate public health measures. Another example is the simultaneous identification of multiple antimicrobial resistance (AMR) genes, which is essential for the optimal management of bacterial infections [12]. Rapid detection of specific AMR genes enables the prescription of targeted, narrow-spectrum antibiotics, thereby improving patient outcomes and facilitating the containment of hospital-acquired infections caused by resistant organisms.

The growing demand for multiplex molecular diagnostics reflects the complex nature of infectious diseases and the necessity for more integrated, rapid, and scalable diagnostic platforms. Such innovations are pivotal for enhancing clinical decision-making, optimising antimicrobial stewardship, and strengthening public health responses to infectious disease outbreaks [3], [12], [13].

To meet these clinical needs, multiplex molecular diagnostic strategies have been developed, where multiple nucleic acid targets are amplified and detected within a single reaction. While conventional multiplex PCR requires separate reactions in numerous wells, which increases costs and sample requirements, advancements in single-well multiplex PCR technology offer a promising solution [14]. By combining the amplification of multiple targets into a single reaction chamber, single-well multiplex assays significantly reduce reagent usage, minimise the consumption of clinical samples, and expedite the diagnostic process [10]. In these systems, the presence of any target gene within the sample results in amplification, which can be detected by post-amplification methods, such as gel electrophoresis and amplification curves.

However, distinguishing the amplifications of individual targets within a single-tube multiplex assay is technically complex. Apart from the traditional gel-based distinguishment, which relies on significantly different amplicon lengths and suffers from low specificity and high operation complexity [15], numerous other strategies have been explored, including but not limited to melting curve analysis (MCA) [16], multi-spectral detection [17], and modulation of the final fluorescence intensity (FFI) [18], each presenting barriers to routine clinical deployment (we will discuss about their drawbacks later in this chapter). Their limitations underscore the need for a robust, low-cost, and user-friendly approach to single-tube, single-channel multiplex NAAT.

An important concept of this thesis is to use information technologies in tackling the challenges of multiplex PCR and LAMP, named by Dr Rodriguez-Manzano's laboratory as "Data-Driven Multiplexing" (DDM) [19]. Following this idea, our group has introduced Amplification Curve Analysis (ACA), which exploits modern machine-learning algorithms to mine the rich kinetic information embedded in real-time amplification traces of PCR [19]. The first implementation utilised a k-nearest-neighbor (KNN) classifier trained on full amplification curves, successfully differentiating three AMR genes in synthetic templates [19]. Building on this foundation, ACA was combined with melting curve features to create Amplification and Melting Curve Analysis (AMCA), enabling single-tube, single-channel discrimination of nine AMR targets [20]. The method was validated on both synthetic deoxyribonucleic acid (DNA) mixtures and authentic clinical specimens.

Despite its achievements, the current ACA/AMCA still experiences limitations in classification accuracy, robustness against chemical variations, and inter-platform discrepancy [21]. Methodologies for designing optimal assays with significant kinetic differences among targets to maximise ACA performance are also underexplored. Furthermore, preliminary efforts in implementing ACA to LAMP have experienced compromised results due to the complex nature of LAMP reactions and their homogenous real-time outputs [22].

In addition to ACA and AMCA, data-driven approaches enable a variety of algorithm-based workflows for computer-aided multiplex assays. This thesis also explored the use of information techniques in colorimetric LAMP (cLAMP), another category of spatial multiplexing that qualitatively leverages the colour changes to indicate amplifications [23]. cLAMP is ideal for point-of-care (POC) nucleic acid assays because it attains single-copy sensitivity in roughly half an hour while operating at a constant temperature, eliminating the need for thermocyclers and complex readout systems [24]. During amplification, the pH value or metal-ion concentration changes in solution, resulting in shifting visible colours when applied with pH- or metal-indicators [23], [25]. cLAMP detection has been validated for numerous pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [26], [27], influenza A virus [28], *Plasmodium falciparum* [29], *Aspergillus fumigatus* [30], *Campylobacter coli* [31], *Streptococcus agalactiae* [32], hepatitis C virus [33], human immunodeficiency virus (HIV), and human papillomavirus (HPV) [34], using only visual cues.

Visual inspection of cLAMP, however, introduces variability: ambient light, subtle hue differences, and colour-vision deficiencies can all impair accuracy, also undermining epidemiological studies [35], [36], [37].

The thesis is dedicated to addressing the current limitations and drawbacks of ACA and cLAMP readout flow by introducing advanced signal processing and machine learning (ML) methodologies, to enhance molecular diagnostics with state-of-the-art achievements in computer science. Detailed hypotheses, aims, and structures of the thesis will be provided later in this chapter. Still, before that, we will first familiarise readers with the relevant background information and elaborate on the significant challenges for data-driven implementation in multiplexing NAAT.

1.2 Background

Part of the concepts in this section resulted in the following peer-reviewed article:

L. Kreitmann, L. Miglietta, K. Xu, K. Malpartida-Cardenas, G. D’Souza, M. Kaforou, K. Brengel-Pesce, L. Drazek, ‘Next-generation molecular diagnostics: Leveraging digital technologies to enhance multiplexing in real-time PCR’, *TrAC Trends in Analytical Chemistry*, vol. 160, p. 116963, Mar. 2023.

Contribution: data curation, validation, writing-review & editing.

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1.2.1 Quantitative Polymerase Chain Reaction (qPCR)

In 1986, Kary Mullis invented the PCR, an in vitro technology that amplifies DNA molecules into millions of copies of fragments [38]. The process called denaturation first opens the DNA double strands with a high temperature, typically 95°C. The single strands are then hybridised with primers, which are specifically designed DNA oligonucleotides flanking the target sequence, at a temperature of 55-65°C (annealing). By introducing polymerase, an enzyme capable of chaining nucleotides and generating new identical molecules, initial templates are amplified starting from the primers, leading to the extension of the new DNA strand, resulting in a process known as elongation or extension.

Theoretically, the number of DNA molecules will double each time a PCR cycle is completed, but eventually the reaction will be saturated due to the depletion of reagents and the equilibrium reached among competing chemical processes [39]. Typically, a PCR experiment

is designed to complete within 40-50 cycles to minimise the generation of secondary products [39].

In addition to traditional post-PCR analysis, such as gel electrophoresis, modern PCR utilises fluorescence to indicate the progress and outcomes of PCR reactions. To achieve this, fluorescent labels that interact with amplicons are required. Two major categories of these labels are intercalating dyes and fluorescent probes [14], [40].

Intercalating dyes, such as SYBR Green I, preferentially intercalate themselves into double-stranded DNA (dsDNA) but display negligible affinity for single-stranded DNA (ssDNA). Upon intercalation, the dye's fluorescence quantum yield increases dramatically because rotational quenching of its conjugated rings is restrained. Consequently, as PCR cycles progress and newly synthesised dsDNA accumulates, the emitted fluorescence intensity increases proportionally to the product concentration, enabling real-time quantification. Because the signal originates from any dsDNA present in the tube, the non-specific binding of intercalating dyes necessitates rigorous primer design and usually requires post-amplification melt-curve analysis to distinguish target amplicons from off-target products or primer dimers [41].

Fluorescent probes, e.g., TaqMan probes, are short, sequence-specific oligonucleotides that carry a reporter dye at the 5' terminus and a quencher moiety at the 3' end [42]. In the intact probe, fluorescence is suppressed through Förster resonance-energy transfer (FRET) or contact quenching. During PCR, the probe anneals to its complementary region within the amplicon. As Taq DNA Polymerase extends the upstream primer, its intrinsic 5'→3' exonuclease activity cleaves the bound probe. This hydrolytic event physically separates the reporter from the quencher, abolishing quenching and allowing the reporter to emit light. Because each newly formed strand can liberate a fluorophore in subsequent cycles, fluorescence rises proportionately to target copy number, enabling target-specific real-time quantification.

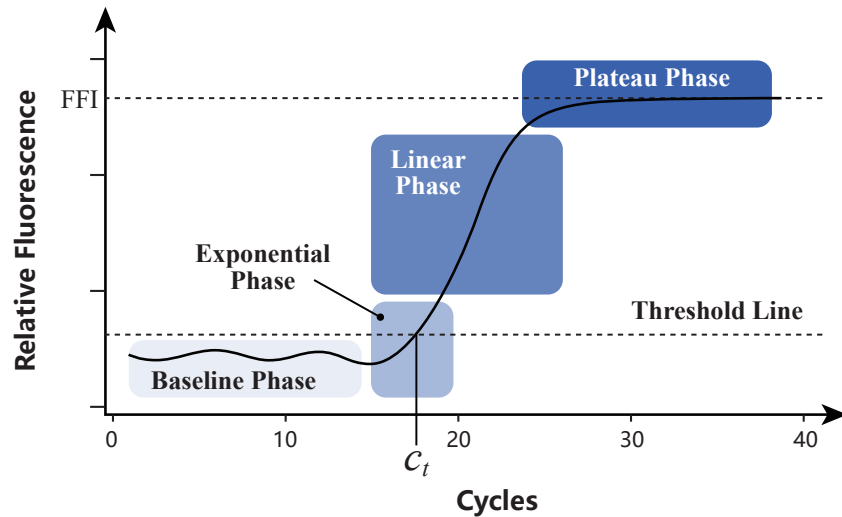


Figure 1.1: Typical structure of an Amplification Curve (AC). Baseline, exponential, linear, and plateau phases are presented in the AC. The fluorescence threshold defines the C_t value, the cycle at which fluorescence exceeds background, marking the onset of exponential DNA amplification. As reagents deplete, the reaction enters a linear phase and eventually reaches a plateau, where the Final Fluorescence Intensity (FFI) reflects the concentration of primers and probes. Adapted from [8] under CC BY 4.0 [43].

By reading out the fluorescence value at the end of each amplification cycle with a fluorescence-based reader, real-time outputs of real-time quantitative PCR (qPCR) can be depicted with a continuous curve called “Amplification Curve” (AC) [44]. A typical structure of AC is illustrated in Figure 1.1, which presents four reaction phases that form a sigmoidal-like shape. By setting a fluorescence threshold, the baseline phase is segmented, representing the initial period of the reaction where newly released fluorescence can barely be distinguished from the background noise. After the threshold, whose corresponding cycle in the X-axis is named cross-threshold (C_t) value, the curve enters the exponential phase where DNA amplification reaches almost 100% efficiency, and the number of amplicons nearly doubles each cycle. This ideal reaction condition is gradually broken by the reduced amount of reagents and competition of other chemical reactions, leading to a linear phase where amplification efficiency drops by cycle numbers [45], [46]. Finally, the reagents are used up and reactions tend towards equilibrium, resulting in a plateau in AC with the fluorescent value at the end of the cycle named Final Fluorescence Intensity (FFI), which is strongly related to the initial concentration of primers and probes [47]. Conventional speaking, limited information can be extracted from AC, as various chemical-related, equipment-related, and human-related factors can influence the readouts [45]. The C_t value, proven exponentially correlated with the initial template concentration [48], was one of the few examples of features obtained from AC that appear to be relatively robust against variations. Our group leveraged emerging machine

learning and deep learning techniques to harness the previously unrevealed power of AC in target identification. However, challenges still exist in manipulating this power.

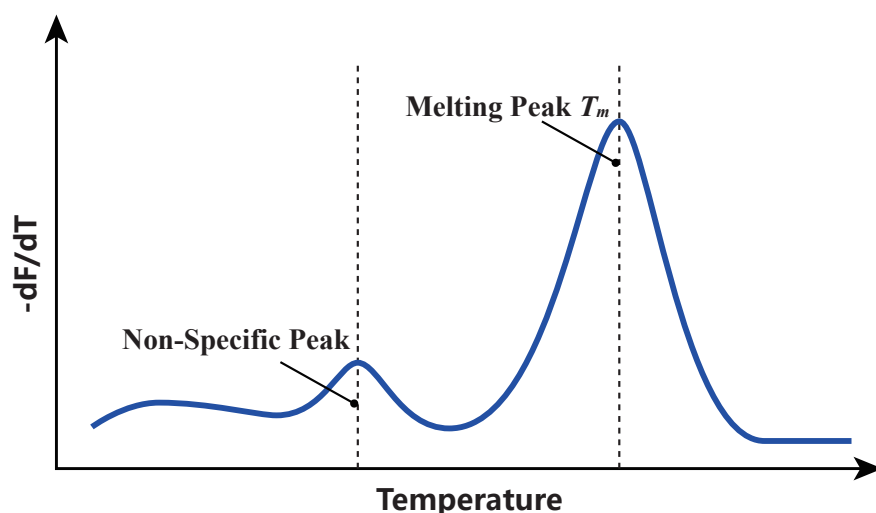


Figure 1.2: Typical structure of a Melting Curve (MC). $-dF/dT$ in the Y-axis label indicates the first derivative of the recorded melting fluorescence versus temperature. Target-specific melting peaks and non-specific peaks can be distinguished by temperature, enabling the multiple target identification using melting analysis. Specific and non-specific products can also be distinguished by their distant melting peak locations.

The MCA, on the other hand, is conventionally generated after the reaction finishes. By gradually heating or cooling the solution and spanning the denaturation temperature range in the presence of an intercalating dye, fluorescent values are recorded at each temperature step [49], [50]. When using intercalating dyes for qPCR, fluorescent values decrease as more DNA double strands denature at higher temperatures, allowing the dyes to be released again into the solution. Since the A-T pair in DNA strands has two hydrogen bonds and the G-C pair has three bonds, amplicons with higher G-C pair percentages and longer strands tend to show higher denaturation temperatures. By calculating the first derivative of the recorded melting fluorescence versus temperature, which is usually called the melting curve (MC), different peaks at various temperatures depict the spectrum of amplicons in the solution, showing both specific reaction products and side products such as secondary structures and primer dimers [51], as shown in Figure 1.2. Later in this chapter, we will discuss state-of-the-art methodologies that utilise melting peaks to distinguish target DNAs. Apparently, MCA is not applicable for probe-based chemistry, where fluorescence does not change upon denaturation, as the fluorophores are already released into the solution during elongation and their concentration will not change when heating.

1.2.2 Quantitative and Colorimetric Loop-Mediated Isothermal Amplification (qLAMP & cLAMP)

First reported by Notomi et al. in 2000 [52], LAMP offers an alternative NAAT solution that enables DNA amplification at a constant temperature. LAMP dispenses with thermal cycling by exploiting the strand-displacement activity of enzymes such as *Bacillus stearothermophilus* (Bst) DNA Polymerase [53], [54]. Using four core primers, including the outer pair (F3, B3) and the inner pair (FIP, BIP, each containing two target-specific regions F1, F2 and B1, B2), the assay quickly constructs a self-priming “dumbbell” structure that supports exponential replication. As depicted in Figure 1.3, the reaction initiates when the F2 segment of FIP replaces the original backward strand and binds to its complementary F2c region. Bst then elongates the new strand while the overhanging F1 segment remains unpaired. Hybridisation of the outer primer F3 releases the initial strand. The same process happens with BIP/B3, generating a complementary product. Subsequent intramolecular pairing of F1/F1c and B1/B1c in the synthesised strand forms the characteristic looped dumbbell, after which every primer can serve as a new start site, generating large, cauliflower-like structures.

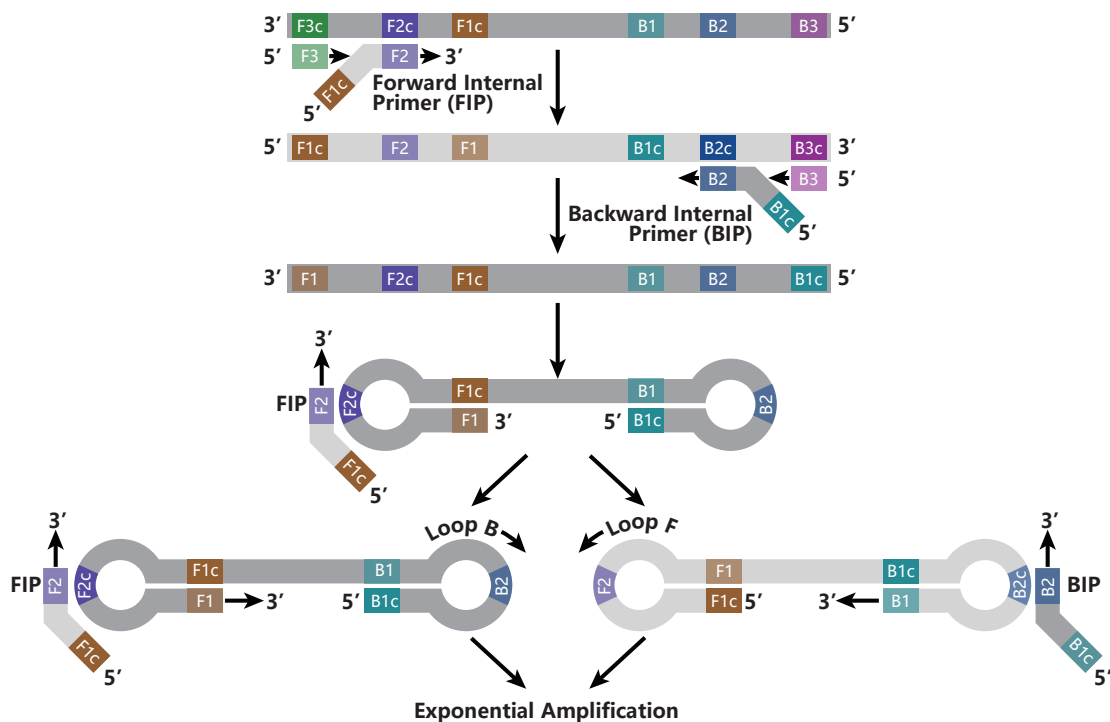


Figure 1.3: Initial steps of LAMP. FIP binds to the F2c region and is extended by Bst Polymerase, while the F1 segment remains unpaired. The binding of F3 then displaces the initial strand. A similar process occurs with BIP/B3, producing a complementary strand. Intramolecular pairing forms a looped dumbbell structure, enabling continuous amplification

and the formation of large, cauliflower-like DNA products. Adapted from [21] under CC BY-NC 4.0 [55].

Because double-stranded DNA accumulates continuously, intercalating dyes permit real-time fluorescence monitoring, enabling the detection of both AC and MC, a process known as real-time quantitative LAMP (qLAMP) [56].

Compared with conventional PCR, LAMP offers practical advantages [52]: the isothermal reaction conditions eliminate the need of thermocyclers, reducing operating complexity and financial costs; the continuous amplification delivers detectable products in less than 30 min, much faster than standard PCR; with six primers targeting a number of distinct regions, it shows higher sensitivity and specificity. Its simplicity, rapid readout, and minimal equipment requirements make LAMP ideal for field diagnostics, especially in resource-limited settings [53].

For example, colorimetric readouts allow for equipment-free detection of results and POC and near-patient applications [57]. cLAMP couples the rapid, high-yield DNA synthesis of conventional LAMP with visual indicators that change hue as the reaction proceeds, eliminating the need for fluorescent optics [56]. During amplification, large quantities of protons and magnesium pyrophosphate are released. These chemical by-products lower the pH and chelate Mg^{2+} ions [23]. By formulating the master mix with pH-sensitive dyes such as phenol red or with metal-ion indicators like hydroxynaphthol blue, the solution shifts in colour when amplification occurs [25]. Because the colour transition is irreversible and robust, results can be read by eye within 20–40 minutes, or captured by a smartphone for objective, algorithm-based interpretation [58]. cLAMP retains the high sensitivity of LAMP while requiring only a constant-temperature heat source, making it especially attractive for point-of-care diagnostics, field surveillance, and low-resource laboratories [59].

In summary, qLAMP and cLAMP provide rapid, sensitive, and user-friendly pathogen detection, which is particularly valuable in resource-constrained or point-of-care settings. A key aim of this thesis is to extend data-driven multiplexing strategies, previously applied to qPCR, to isothermal platforms like LAMP, thereby combining the assay's instrument simplicity with machine-learning-guided target discrimination.

1.2.3 Reverse Transcription for Ribonucleic Acid

Reverse-transcription PCR (RT-PCR) [60] and reverse-transcription LAMP (RT-LAMP) [61] are indispensable tools whenever the target pathogen contains a ribonucleic acid (RNA)

genome rather than DNA. In these assays, the RNA template is first converted into complementary DNA (cDNA) by a reverse transcriptase enzyme, after which standard PCR or LAMP chemistry can amplify the resulting cDNA [62]. This two-step approach combines the high sensitivity and specificity of nucleic acid amplification with the ability to detect RNA-based viruses and other pathogens [60]. Consequently, in all experimental chapters of this thesis, RNA targets were first reverse-transcribed into cDNA, and every downstream PCR or LAMP assay was performed on that cDNA template, except for the synthetic cDNA, which was synthesised by and purchased from manufacturers.

1.2.4 Digital Platform: From Precise Quantification to Big-Data

Empowerment

Different from conventional qPCR, in which the reaction happens in bulk status in μL -level reaction tubes, digital PCR (dPCR) partitions the sample solution into numerous separate sub-reactions occurring in tiny compartments, as depicted in Figure 1.4. Different mechanisms are introduced for the partitions [63]: droplet dPCR (ddPCR) partitions the solution into thousands of demulsified microdroplets suspended in oil, each acting as an independent reactor; microwell array systems contain $n\text{L}$ -level chambers moulded in PDMS or silicon, enabling parallel amplification across discrete wells; microfluidic valving technology integrates multilayer pneumatic valves and pumps on chip to dynamically isolate sub-nanolitre reaction volumes within fluidic networks, providing precise control over partitioning, reagent flow, and thermal cycling. The most significant advantage of dPCR, apart from its reduced template competition and robustness against inhibitors [8], lies in its capability for absolute quantification. As we mentioned in 1.2.1, qPCR quantification typically relies on indirect measurement of other concentration-correlated features, such as C_t values and the standard curve derived, limiting its applications in precise diagnostics and quantitative research. dPCR, on the other hand, splits templates and reagents into individual compartments, ideally resulting in single-molecule reactions with at most one DNA molecule per chamber. In this way, template concentrations can be calculated directly by counting the number of positive microreactors with a fluorescence detection system [64], [65]. Leading dPCR equipment even allows for real-time fluorescence outputs (qdPCR), generating amplification and melting curves for individual micro wells [66], [67], [68].

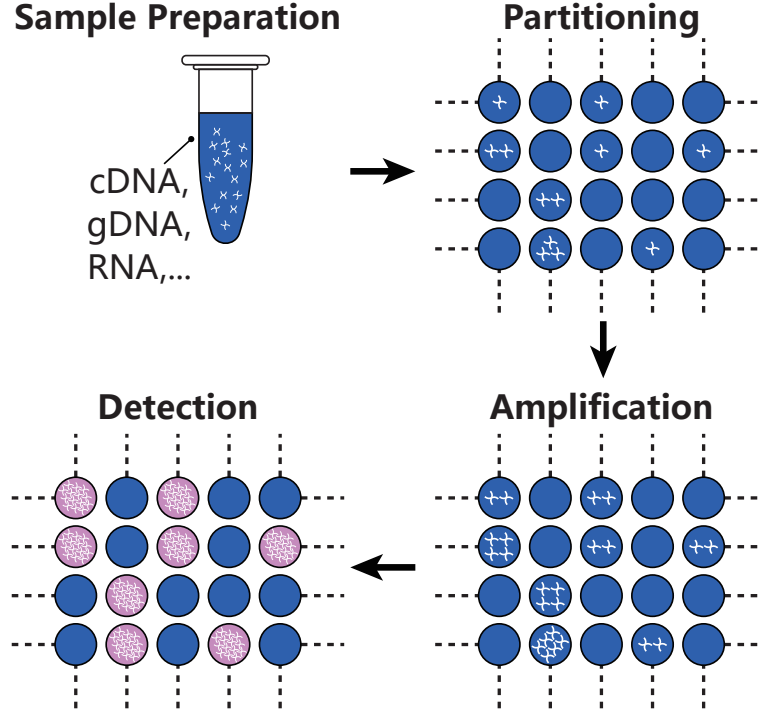


Figure 1.4: Basic principle of dPCR. After sample preparation, the solution is partitioned into hundreds/thousands of micro reaction chambers, with the template numbers in chambers following a Poisson distribution. Targets are then amplified separately, and the final detection and quantification of the sample are conducted by counting the number of positive chambers and applying the adjustment equations. Adapted from [8] under CC BY 4.0 [43].

However, the assumption of “single-molecule reactions” faces challenges when considering the real situation of molecule distributions. In a simplified scenario, M (unknown number) molecules of templates in V volume of solution are split randomly and uniformly into N (known number) partitions. The probability of each partition showing a positive result (including at least one template molecule) is

$$P(pos) = 1 - P(neg) = 1 - \left(1 - \frac{1}{N}\right)^M, \quad (1.1)$$

where $P(neg)$ is the probability of showing a negative result (zero molecule shows up in that partition). When N is large,

$$\lim_{N \rightarrow \infty} P(pos) = \lim_{N \rightarrow \infty} 1 - \left(1 - \frac{1}{N}\right)^M = 1 - e^{-\lambda}, \quad (1.2)$$

in which $\lambda = \frac{M}{N}$. After reactions, $P(pos)$ can be estimated by calculating the proportion of observed positive partitions K to the total number of partitions N . In this way, λ is estimated by

$$\hat{\lambda} = -\ln\left(1 - \frac{K}{N}\right). \quad (1.3)$$

The initial concentration of target templates can then be determined by

$$conc = \frac{\hat{\lambda} \times N}{V}. \quad (1.4)$$

From another perspective, the probability of a partition containing k molecules can be estimated using a Poisson distribution [64]:

$$P(k) = \frac{\lambda^k e^{-\lambda}}{k!}. \quad (1.5)$$

Therefore, dPCR partition counts follow a parametric model, and statistical confidence can be estimated for the inferred copy number. The width of that interval is governed by the number of positive partitions versus negative ones. When nearly every well is positive, or in contrast, when almost all are negative, sampling information is sparse, and the estimate is imprecise. Numerous classical approaches are used to translate the observed positive fraction into a 95% confidence interval, including the most commonly used Wilson or Clopper-Pearson methods [8].

Conventionally, the ideal reaction condition for dPCR is to maintain a concentration of λ around 1.6 [8], reducing the estimation error. However, recent research from our group has extended the usability of dPCR to bulk reactions, where individual partitions are fully saturated with significantly more molecules at the initial concentration [69]. A transition period from digital to bulk status was observed, and standard curves showed C_t dropping linearly with log-transformed concentration increment, similar to those in qPCR. This finding lays the groundwork for our subsequent applications: in this research, dPCR generates millions of reaction real-time outputs of large ranges of template concentrations in a high-throughput way, with moderate time and resource consumption compared with traditional qPCR, which can typically provide only 96 observations per run. Leveraging the large amount of data from dPCR benefits the implementation of sophisticated machine/deep learning algorithms, providing the variability and scalability required for data-driven models [19]. This strategy will, of course, introduce limitations and bias, which are going to be discussed in 1.3.1.

1.2.5 Fundamentals of Multiplex Molecular Diagnostics

Moving from singleplex to multiplex NAAT immediately raises the question of how to tell amplicons that may emerge from the same tube apart. The earliest answer was to let size do the

talking: amplified fragments migrate through an agarose or polyacrylamide matrix under an electric field and separate according to length, so each band on the gel provides a fingerprint of its template [15]. Despite its simplicity in principle and low cost, gel electrophoresis is labour-intensive, yielding information only at the end of the run, and can be less precise in identifying multiple target amplicons of similar size. These limitations encouraged the adoption of fluorescence-based readouts in target identification.

The development of qPCR, and more recently qLAMP, transformed detection by monitoring fluorescence change during the amplification reaction. Dedicatedly designed probes of different targets can be tagged with distinct fluorophores. Modern thermal cyclers are usually equipped with four to six colour channels [14], and a multiplex assay therefore assigns each target-probe pair a unique spectral address and tracks their AC or FFI simultaneously. Although this colour-coded approach is fast and quantitative, its multiplexing ceiling is fixed by the number of optical channels and also by spectral crosstalk between dyes [70], so a finer way of discrimination is still needed when dozens of genetic signatures must be resolved at once.

An alternative way to solve this is by using MCA. As we introduced in 1.2.1, after amplification, the reaction is heated gradually in the presence of dye, and fluorescence is recorded at every increment of temperature as double-stranded DNA melts into single strands. The resulting melting curve reflects the stability of the duplex, which depends on its length and G-C content. Plotting the negative first derivative of the curve pinpoints the melting temperature peak T_m , at which half the molecules are dissociated, and the temperature is dropping at its highest rate [71]. Instruments capable of precise temperature control generate high-resolution melting profiles, and even a single-nucleotide difference is enough to shift the peak location [72]. This intrinsic thermodynamic signature allows a single optical channel to distinguish a large panel of products, enabling applications that range from subtyping influenza and coronaviruses [73], [74] to broad bacterial identification, such as variable ribosomal loci and resistance mutations in *Neisseria gonorrhoeae* [73]. When high-resolution melting is combined with microfluidic chips or digital PCR arrays, spatial partitioning and sequence-level fingerprints reinforce each other and push multiplex capacity further [75]. New techniques are also emerging to generate distinct melting peaks for easier target identification. For example, a single-tube multiplex asymmetric PCR-high-resolution melting assay was proven to accurately genotype key folate-metabolism SNPs from as little as 1 ng of blood-derived DNA, showing clinical-grade sensitivity, speed, and contamination resistance while avoiding the high cost and complexity of sequencing-based methods [76]. In addition, Zhang et al. [16] introduced the dynamic melting curve, which collects fluorescent changes during the denaturation of the

standard PCR process with a carefully designed temperature changing rate, presenting melting peaks and distinguishing targets simultaneously during PCR cycles.

Extracting reliable answers from melting data now hinges on sophisticated signal processing, rather than human inspection. The signal-processing workflow follows a familiar sequence [77]: baseline fluorescence is subtracted, and values are normalised. Traces are then ‘temperature-shifted’ so that minor thermal offsets among wells or runs are aligned. Next, hierarchical clustering groups similar traces. Difference plots are then produced by subtracting each cluster’s fluorescence from that of a reference. Finally, Savitzky-Golay polynomial estimation is applied to compute negative first derivative plots of normalised melting data [78].

1.2.6 Data-Driven Approaches in Multiplexing: State-of-the-Art

1.2.6.1 Data-Driven Multiplexing for Melting Curves

Although the term “Data-Driven Multiplexing” (DDM) was first coined in 2020 by Moniri et al. [19] from our group, the concept of using machine learning, unsupervised or supervised, to mine the implicit power of numerous real-time data in multiplex reactions has long been adopted and implemented in MCA. While traditional signal processing operations can cluster curves from identical amplicons, the large-scale identification of unknowns requires approaches that extend beyond manual review or the basic clustering tools typically bundled with most instruments. Recent studies have therefore turned to machine learning for MCA. Athamanolap et al. 2014 simulated melting curves for the *cps* locus of 92 *Streptococcus pneumoniae* serotypes, trained an ensemble of linear-kernel support-vector machines (SVM), and achieved an average accuracy of 99.9% [79]. The same group later compiled an experimental library of >1000-bp 16S amplicon curves from 37 bacterial species, training a nested SVM that performed well with cultured isolates but less robustly on clinical specimens [80]. More recently, convolutional neural networks have been deployed to classify high-resolution melting data after converting each curve into a recurrence-plot image [81].

1.2.6.2 Data-Driven Multiplexing for Amplification Curves

However, when migrating machine learning algorithms into amplification curves, it becomes less straightforward. It is partly because of the internal complexity of AC shapes that is influenced by various factors, including equipment characteristics, reagent consistency, and differences in sample-extraction methods [82], [83], leading to dynamically changed reaction efficiencies, reagent volume, and fluorescent-readout linearity. In addition, AC may not show apparent inter-target discrepancy during visual inspection in unoptimised assays, due to the

modulation of reaction information implicitly hidden inside the continuous data points of curves throughout the entire reaction period, which is far more abstract than T_m in MCA. Furthermore, learning on continuous AC shapes, whether using feature extraction plus statistical learning or end-to-end deep learning, requires effective condensation of kinetic information with hand-crafted or automatic features, which is a significant problem when considering the relatively high AC dimension of 40-50. Last but not least, like all the data-driven approaches, DDM expects a large amount of training data with variations covering factors in real-clinical scenarios, for building robust and highly generalisable models.

As we mentioned in 1.2.4, the emerging dPCR technology has the potential to generate thousands of ACs in a single experiment, especially when the reactions are formulated to run in bulk format. This prompted our exploration of applying DDM on AC.

The initial breakthrough occurred when we recognised that traditional AC features could be combined in a single reaction to build multidimensional standard curves (MSCs) [84], [85]. By capturing physical characteristics of the amplification process within an analytical framework, MSCs made it possible to flag non-specific products as outliers and to enhance the accuracy of DNA quantification. More importantly, MSC revealed the hidden relationship among different DNA targets when representing them in the high-dimensional space of AC. Visual inspection confirmed the distinct data clusters of targets when plotting the feature vectors in 3D. It was only natural to turn to machine learning algorithms to distinguish these targets.

The concept of “Amplification Curve Analysis” was officially introduced by Moniri et al. 2020 [19]. By applying KNN models on the time series of amplification curves, ACA was proven effective in distinguishing three common carbapenem-resistant genes in a controlled multiplex lab setting on synthetic DNA. This research provided a new perspective on leveraging kinetic information in AC, and inspired numerous related works. For instance, Yao et al. 2022 developed an algorithm for identifying positive reactions from thousands of dPCR real-time outputs, which appear to be noisy with notches and complex in shape due to the variance of digital reactions [86]. By combining Principal Component Analysis (PCA) extracted features from AC with a Fisher classifier, they reduced the recognition error of positive wells by 64.6% with low-concentration samples. Another group analysed and predicted the kinetic information of AC time series with deep learning techniques [87]. They used GRU networks for SARS-CoV-2 ORF1ab detection, predicting positives after only 13 cycles and endpoint values by 20 cycles, thereby cutting testing time by roughly 67%.

To extend the multiplex number of ACA, information from melting curves was also encoded and merged into the machine learning workflow. ACA and MCA both examine the same

amplicon, yet they tap into complementary layers of information. The real-time amplification output records kinetic behaviour, presenting how efficiently the reaction proceeds from cycle to cycle, whereas the post-run melting profile reflects thermodynamic stability, which depends on attributes such as G-C content and fragment length [71]. Unifying these two readouts combines kinetic and thermodynamic signatures, providing a more powerful basis for differentiating targets than either curve can offer on its own. The initial effort was contributed in [20], where the concept of AMCA was introduced. By proposing a new workflow, in which the probability predicted by a two-layer Convolutional Neural Network (CNN) on AC real-time data was concatenated with the output probability of a logistic regression learner trained on melting curves, this work demonstrated the AMCA's efficacy in handling complex multiplex conditions, showing a classification accuracy over 99.3% on differentiating nine mobilised colistin resistance genes with synthetic templates. The effectiveness of AMCA was further validated in our subsequent publication [69], where AMCA demonstrated satisfying classification performance on 253 clinical isolates, with 99.6% accuracy on 160,041 reactions. Another example of utilising AC and MC in DDM was presented by Godmer et al. [88]. In their research, ten kinetic features related to the first and second derivative curves of AC were extracted and combined with four features from the denaturation plot. The feature vector was then fed into a Random Forest (RF) classifier to predict positive events.

Although AMCA has become a powerful way to fingerprint amplicons, its application is restricted by the hardware demands. Precise thermocyclers are required to generate finely stepped temperature ramps and capture fluorescence at every increment. Still, these are absent in many point-of-care instruments, especially those based on isothermal chemistry. In addition, assays that rely on probe chemistries (e.g., TaqMan panels) lack the intercalating dye required to generate melt curves. Furthermore, MCA separates amplicons by distinct melting temperatures; therefore, primer sets must be carefully engineered to create non-overlapping T_m peaks. Even with such optimisation, crowded peaks typically limit reliable discrimination to targets.

In contrast, because ACA is compatible with various reporter chemistries, it works with intercalating dyes, hydrolysis probes, and even digital LAMP droplets, thereby sidestepping the “no-melt-curve” problem in probe-only assays. Therefore, this thesis will focus on improving ACA, from the assay design and cross-domain classification to its adaptation in LAMP.

Although demonstrated to be feasible in classifying five respiratory pathogens using a digital platform [22], research on implementing ACA in LAMP has been hindered, mostly due to the complex nature of continuous reactions and products. This will be further discussed in 1.3.5.

1.2.6.3 Assay Design for Amplification Curve Analysis

As ACA analyses the kinetic process of real-time output, it is natural to consider designing assays that generate distinct amplification shapes, enabling easier target differentiation. The most apparent feature of amplification curves lies in the plateau phase, in which the FFI can be extracted for target identification. In this thesis, attempts to manipulate the FFI for better distinction are referred to as “FFI modulation.”

Although intercalating dye-based reactions may illustrate FFI differences among amplicons of varying lengths, it is not practical to utilise these subtle discrepancies for target identification, considering their less robustness to reagent variations and the potential requirement for redesigning amplified regions on targets. In contrast, probe-based chemistries like TaqMan can theoretically provide changeable final fluorescence by modifying probe initial concentrations, which determine the total reporters released when probes are fully consumed. The first effort in this field was made by Zhong et al. in 2011, who demonstrated that distinct fluorogenic probes sharing the same spectral channel can be differentiated by deliberately adjusting their concentrations, allowing each to produce a characteristic fluorescence amplitude [89]. By introducing additional dye colours, they multiplied the number of probe-intensity combinations, validated in a five-plex spinal muscular atrophy assay. Recent studies have concentrated mainly on the increase in target numbers and clinical applications, exemplified by a series of investigations conducted by ChromaCode Inc. In their first work, TaqMan probe concentration-based fluorescent amplitude modulation was performed with three colour channels in measuring and identifying nine targets (note that each target was detected by only one probe of one colour channel), demonstrating an over 98.9% accuracy [47]. Examples of co-amplification in single channels were also provided [47]. Later, they extended their work into high-definition PCR (HDPCR), in which they incorporated two channels for encoding each target, also with various probe concentrations, resulting in a 2D plot of targets and target co-amplification combinations that can be visually distinguished or clustered [18]. Clinical proofs in detecting DNA/RNA targets of non-small cell lung cancer in 62 formalin-fixed paraffin-embedded human samples further highlighted its potential in clinical diagnosis [90]. Special attention has also been paid by Zhang et al. to change FFI by introducing both a probe-based technique and intercalating dyes to increase plateau differences between targets [91].

FFI modulation offers a simplified approach for target differentiation in multiplex amplification assays, but optimising the shape of the whole amplification curve remains essential. FFI captures only the endpoint signal and lacks kinetic context, potentially missing the early-phase dynamics that are crucial for distinguishing closely related targets. Whole curve

analysis provides comprehensive kinetic information, including lag time, exponential rate, and plateau behaviour, which are vital for specificity. Moreover, FFI is susceptible to artefacts such as non-specific amplification or signal saturation, which can lead to false positives. Curve shape features help differentiate genuine amplification from such anomalies. Additionally, machine learning models benefit significantly from temporal data across the curve, enabling finer classification than endpoint values alone.

Changing amplification curve shapes requires sophisticated chemical techniques in tuning reaction efficiencies. Digitised Kinetic Analysis by Park et al. demonstrated a possible solution, leveraging the inherent efficiency shift of single-base mismatch in PCR reactions [92]. By calculating the maximum slopes (which are strongly related to efficiency) of AC, they proved the statistically significant differences in AC shapes between wild-type and Omicron RNA of SARS-CoV-2. However, the drawback is evident: a mismatch must be designed that is not only troublesome but also inhibits the reaction, leading to delayed or unreliable amplification. Similar problems also exist for asymmetric PCR-based shape modulations [93].

An alternative approach to increasing the distinction between amplification curves of different targets is to select distinct shapes of curves deliberately. With a number of candidate singleplex assays designed for each target, multiplex in wet-lab experiments can be carried out on all the possible assay combinations to select the one that generates the largest inter-target curve distances. Apparently, the number of combinations to test increases exponentially with target numbers, making it impossible to test all candidates in multiplex empirically.

The Smart-Plexer workflow, an in-silico pipeline that speeds up multiplex-PCR design by computationally selecting optimal primer mixes before any large-scale bench work, was introduced by our group to tackle this issue [94]. The workflow starts with numerous candidate primer sets per target, running each set in singleplex individually, and recording the resulting amplification curves. It then simulates how those singleplex kinetics behave in a multiplex context, assuming that the underlying reaction dynamics remain consistent during the transition. A numerical feature, parameter ‘*c*’, that remains strongly correlated when transferring from singleplex to multiplex, is extracted from AC. Pairwise distances between targets are computed based on the feature, resulting in two scores: the Average Distance Score (ADS), which reflects overall separation among targets, and the Minimum Distance Score (MDS), which indicates the narrowest gap. Mixes with large ADS and MDS values rank highest, and only these top candidates are taken to the lab for validation, considerably cutting experimental effort and cost. Together with other multiplex design tools, such as Simulated Annealing Design using Dimer Likelihood Estimation (SADDLE), which eliminates potential primer dimers [95], Smart-

Plexer has great potential in expanding the uses of the ACA method while significantly enhancing its adaptability and scalability. However, the current version of Smart-Plexer faces great challenges in real-world scenarios, which will be discussed later in 1.3.2.

1.2.6.4 Data-Driven Approaches in Colorimetric LAMP

As mentioned in 1.1, another primary focus of this thesis is to introduce data-driven approaches in cLAMP. cLAMP replaces fluorescent optics with a simple hue change, but its chief strength, direct visual inspection, also becomes a liability. Colour judgments vary with ambient light, camera angle, and observer bias. They may fail altogether for people with colour-vision deficiency (approximately 8% of men and 0.4% of women in Europe [35]) and become unreliable when the hue shift is slight. These limitations erode the sensitivity and specificity needed for rigorous epidemiological surveys. Capturing reactions with a smartphone or tablet camera offers a low-cost remedy. Digital images can be analysed automatically, improving detection at low template loads, standardising readouts across sites, and streaming results to cloud databases and e-health records [59].

However, turning that idea into practice confronts its own technical hurdles, which will be discussed in 1.3.4. While smartphone-enabled automation can transform cLAMP into a robust, networked diagnostic, further work is needed to balance accuracy, cost, and portability across real-world conditions.

1.3 Challenges for Data-Driven Approaches in Multiplexing

Since the aim of this thesis is to enhance the existing data-driven workflows of Multiplex NAAT for Infectious Disease diagnostics with signal processing and machine learning techniques, it is crucial to define the current issues and challenges.

1.3.1 Artefacts in Amplification Curves

In any machine-learning framework that decodes multiplex reactions from real-time kinetics, the reliability of ACA hinges on the integrity of the curves themselves. Unfortunately, multiplex PCR is prone to artefacts, including the formation of primer-dimer, low-level amplification of untargeted loci, miscalibration of the instrument, and secondary structures of templates, which can all distort an AC that would otherwise exhibit a smooth, high-efficiency sigmoidal profile [48]. Such abnormalities manifest as delayed exponential phases, plain slopes, or irregular plateaus. These kinetic distortions mask the subtle, target-specific features that ACA relies on [19]. From the perspective of machine learning, when curves from different targets are

projected into the feature space, these deviations compress inter-cluster distances and broaden cluster boundaries, making the classification algorithm struggle to separate true targets from one another, driving down overall accuracy.

AC distortion is especially pronounced on digital molecular platforms because each partition contains only a number of templates, and even minor aberrations dominate the kinetics recorded from that partition. A single primer-dimer or polymerase stall in a droplet can lengthen the reaction phase or flatten the exponential slope [96]. The high surface-to-volume ratio in chips and emulsions further aggravates the problem: primers and polymerase adsorb to channel walls or oil interfaces, diluting reagent concentrations and lowering reaction efficiency [97]. Thermal non-uniformity adds another layer of variability: microfluidic substrates can exhibit gradients of $\geq 1^\circ\text{C}$ across the array, slowing reactions in edge partitions and shifting C_t values [98]. Instrument issues such as camera edge distortion also contribute to the abnormality observed [99]. Together, these stochastic, physicochemical, and instrumental factors make digital platforms far more susceptible to distorted amplification curves than conventional bulk reactions.

Therefore, before further analysis can be carried out, a robust and unsupervised methodology is required for eliminating non-specific and low-efficiency reactions from any real-time data used.

1.3.2 Challenges of Optimal Assay Selection

Smart-Plexer proposes a practical workflow for in-silico selection of multiplex assays. However, the current version of Smart-Plexer (Smart-Plexer 1.0) extracts a single kinetic descriptor, the slope-related parameter ‘ c ’ from a five-parameter asymmetric-sigmoid fit, to measure how far apart targets lay in the feature space. Although that unique metric was proven stable in clean singleplex reactions, it can be perturbed in real-world multiplex assays where inhibitors, unequal primer efficiencies, or fluctuating template loads alter reaction efficiency, shift plateaus, and distort curve slopes. These distortions change ‘ c ’ values and the calculated inter-target distances, weakening the method’s discriminatory power.

A second limitation of Smart-Plexer 1.0 comes from the way those inter-target distances were generated. It ranks primer mixes by the median (average) separation between cluster centroids, ignoring how widely individual amplification curves are scattered within each cluster. Overlapping or highly variable clusters could therefore receive deceptively large ADS and MDS, masking poor practical separability.

Therefore, the reliance on a single feature and the use of centroid-only statistics constrain the Smart-Plexer 1.0's robustness and multiplexing accuracy.

1.3.3 Cross-Domain Issue: From Laboratory to Clinics

Although earlier studies underscored the potential of the ACA, two key obstacles remain. First, studies have been underlying the dynamics of PCR/LAMP amplification curves and utilising mathematical models to depict the process [82], [83], [100], [101], yet extracting hand-crafted reliable descriptive features for ACA requires more exploration. There is an increasing need for an end-to-end approach that learns its own representations directly from the raw fluorescent trace, to enable efficient DDM.

Second, curve distributions obtained from synthetic templates in the absence of sample background differ significantly from those recorded in clinical specimens, where the target genome is mixed with human DNA and a variety of commensal or pathogenic organisms [102]. In human pathogen diagnostics, different sample sources, such as blood, saliva, stool, urine, or sputum, can change real-time PCR amplification curves, leading to shifted C_t , reduced slopes, or lower FFI, by altering polymerase activity and fluorescence detection [103]. Therefore, apparent curve differences may reflect the matrix. While synthetic DNA is inexpensive and easy to acquire for model building, annotated clinical samples can be difficult to obtain, especially during the emergence of new pathogens, where safety and ethical consent need to be considered. Ideally, one would train a deep ACA classifier rapidly on synthetic template data and deploy it directly on unlabelled patient samples. However, in practice, models tuned on the synthetic "source" domain perform far worse on the clinical "target" domain than internal cross-validation suggests. From a machine-learning perspective, this reflects a domain shift [104].

In addition, domain discrepancies arise from other factors, such as the variance in chemical agents, operational procedures, and manufacturer bias. They also exist when transferring a large model trained with data from digital platforms to conventional real-time equipment. Therefore, it is our priority to seek a universal approach of deep transfer learning to mitigate domain shifts in an unsupervised manner, without needing labels from the target domain.

1.3.4 Automatic cLAMP in Uncontrolled Environments

Automating cLAMP visual readout is complicated by environmental variability, including changing ambient light, reflections, and the dependency on customised cameras or sensors [58]. Current solutions fall into three broad categories: (1) Custom-designed instruments that integrate incubation heaters with on-board imagers [105] or spectral sensors [27], [28], [32].

Since the optical path and lighting are fixed inside a closed chamber, these systems deliver reliable colour interpretation, but their dedicated components drive up cost and limit use at resource-restrained POC sites. (2) The “semi-portable” route in which smartphones or tablets are used inside controlled holders or observation points [26], [30], [31], [33], [58], [106], [107]. When illumination and tube position are standardised, simple thresholding or radiometric indices can classify colours, yet the algorithms are tied to that specific fixture and lighting preset. (3) Fully portable device-based setups were proposed for non-controlled environments, relying on advanced processing algorithms instead of specific hardware setups [108], [109]. Here, advanced computer vision-based deep learning has been involved, but these models demand large, context-specific training sets. Their “black box” nature also raises interpretability and regulatory concerns [110], [111]. There is a need for a universal, automated colour detection method to improve ease of use and reduce readout subjectivity using portable devices.

1.3.5 ACA in qLAMP: Why Is It More Difficult?

Compared to PCR, LAMP produces fluorescence curves that rise earlier and look almost identical across different targets. A demonstration of digital outputs for 3-target multiplexing in PCR and LAMP is shown in Figure 1.5. The three targets in PCR are showing significant morphological differences in beginning, maximum, and ending slopes, as well as in FFI. In contrast, LAMP outputs exhibit similar slope information due to relatively faster reactions, making them harder to distinguish from ACA. Two mechanistic reasons explain the earlier appearance of the signal. First, LAMP operates continuously at a single temperature, so polymerase never pauses for denaturation and annealing steps; the strand-displacing Bst enzyme can extend and displace in the same stroke without fixed thermal cycling. Second, each target is detected by four to six primers that form looped structures; once the first few dumbbell templates are generated, they self-prime endlessly, creating rapidly branching. As a result, fluorescence often reaches a plateau in 30 min, whereas PCR usually requires 40-50 cycles over approximately 60-90 min [52].

The visual similarity of curves from different LAMP targets also comes from the reaction's architecture. Because every assay uses the same primer topology (F3/B3 outer primers, FIP/BIP inner primers, and optional loop primers), the kinetics are dominated by enzyme speed rather than by individual primer-pair efficiencies, G-C content, or amplicon length variables that create different sigmoidal shapes in PCR [48]. In LAMP, once the self-priming loops take over, minor sequence-dependent lags in the initiation phase are quickly masked by the massive downstream amplification. Consequently, while PCR curves often carry informative kinetic

signatures that differ from target to target, LAMP curves show limited diversity, making target discrimination more challenging.

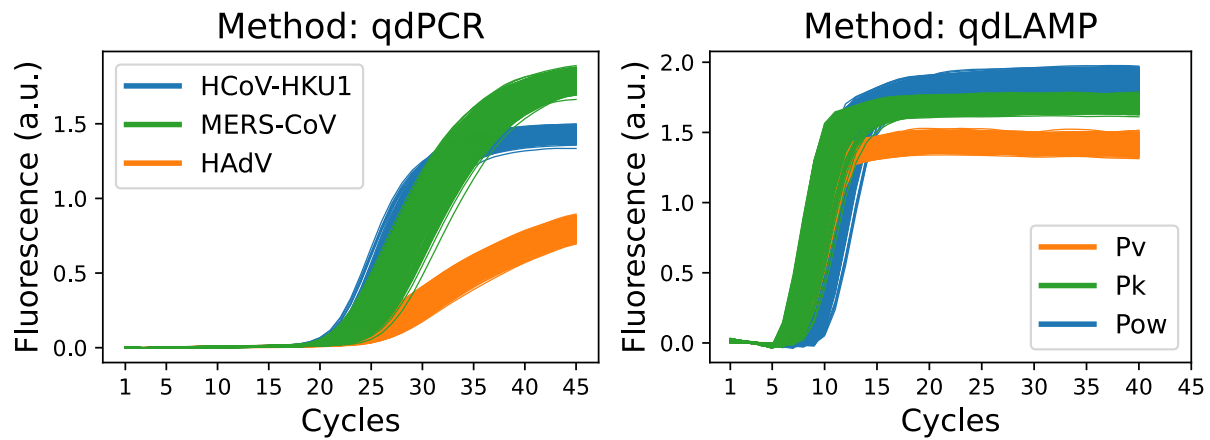


Figure 1.5: A demonstration of digital outputs for 3-target multiplexing in PCR and LAMP. Full names of targets are described in the List of Abbreviations. The three targets in PCR are showing significant morphological differences in beginning, maximum, and ending slopes, as well as in FFI. LAMP outputs exhibit similar slope information due to relatively faster reactions, making them harder to be distinguished with ACA.

1.4 Hypothesis and Aims

To address the aforementioned challenges and unlock the full potential of data-driven approaches in the context of multi-pathogen detection, this thesis proposes the following research hypotheses and detailed aims.

HYPOTHESIS

Integrating advanced signal processing and machine learning with real-time and colorimetric molecular multiplexing methods has the potential to enhance the throughput, accuracy and diagnostic performance of multi-pathogen detection for infectious diseases.

AIMS

- (1) **Adaptive filtering for the digital platform:** To leverage the power of millions of data points from the digital platform and further improve ACA performance for dPCR, a method needs to be developed for identifying and filtering out non-specific and low-efficiency reactions by looking at amplification curves.
- (2) **New features leading to optimised single-well multiplex assays:** Biologically meaningful and experimentally robust features should be extracted from amplification

curves. Along with new distance measurement and Smart-Plexer Strategies, these will guide the optimisation of PCR/LAMP assay selection and improve the classification performance of DDM methods.

- (3) **Domain adaptation for cross-domain applications:** To further improve the cross-environment robustness of ACA, we need to leverage the millions of data points generated by real-time digital platforms to train advanced machine/deep learning models, and adapt them to different application scenarios (e.g., different equipment/platforms, chemical settings, DNA sources).
- (4) **Automatic readout flow for cLAMP:** To demonstrate the utilisation of state-of-the-art information techniques in colorimetric molecular diagnosis, a fully automated readout flow for cLAMP reaction results should be proposed and further tested in real-world clinical applications to verify its capability.
- (5) **Time-to-Positive (TTP) modulation of real-time LAMP for enhanced ACA:** Considering the similar morphological shapes of AC in qLAMP, computationally guided assay design methodologies with chemical modifications are needed. This thesis aims to introduce a new methodology for modulating real-time LAMP outputs by changing TTP ranges of different targets to minimise range overlapping among targets, for precise ACA in qLAMP.

1.5 Thesis Structure and Contributions

1.5.1 Thesis Structure

The structure of the thesis is presented in Figure 1.6. According to the hypothesis and research aims, this thesis will focus on enhancing the current diagnosis flow of fluorescence-based and colorimetric NAAT. After conducting a comprehensive literature review and outlining the specific challenges in Chapter 1, the research journey begins by purifying the numerous amplification data from digital platforms using the Adaptive Mapping Filter proposed in Chapter 2, thereby establishing a reliable and extensive data foundation for the subsequent research. With solid data, Chapter 3 focuses on enhancing the computer-aided in-silico assay development flow, which enables the precise selection of ACA-optimal primer mix, also reducing the experimental testing workload. Built on these processing and design selection tools, Chapter 4 proposes a deep domain adaptation network dedicated to ACA, and explores its efficacy in improving DNA target identification in complicated clinical scenarios of data-source

shifting, demonstrating the improved ACA accuracy, which may be compatible with clinical requirements. Chapter 5 further extends data-driven approaches in cLAMP, illustrating a reliable automated readout framework for cLAMP multiplexing that utilises advanced image processing and deep learning techniques, thereby further supporting the thesis hypothesis. Finally, preliminary research in Chapter 6 puts efforts into migrating the ACA technology to qLAMP-based single-well multiplexing. Chapter 3 and Chapter 6 deal with the topic of assay optimisation for real-time PCR and LAMP, respectively, from the perspective of either selecting optimal combinations or designing assays in silico.

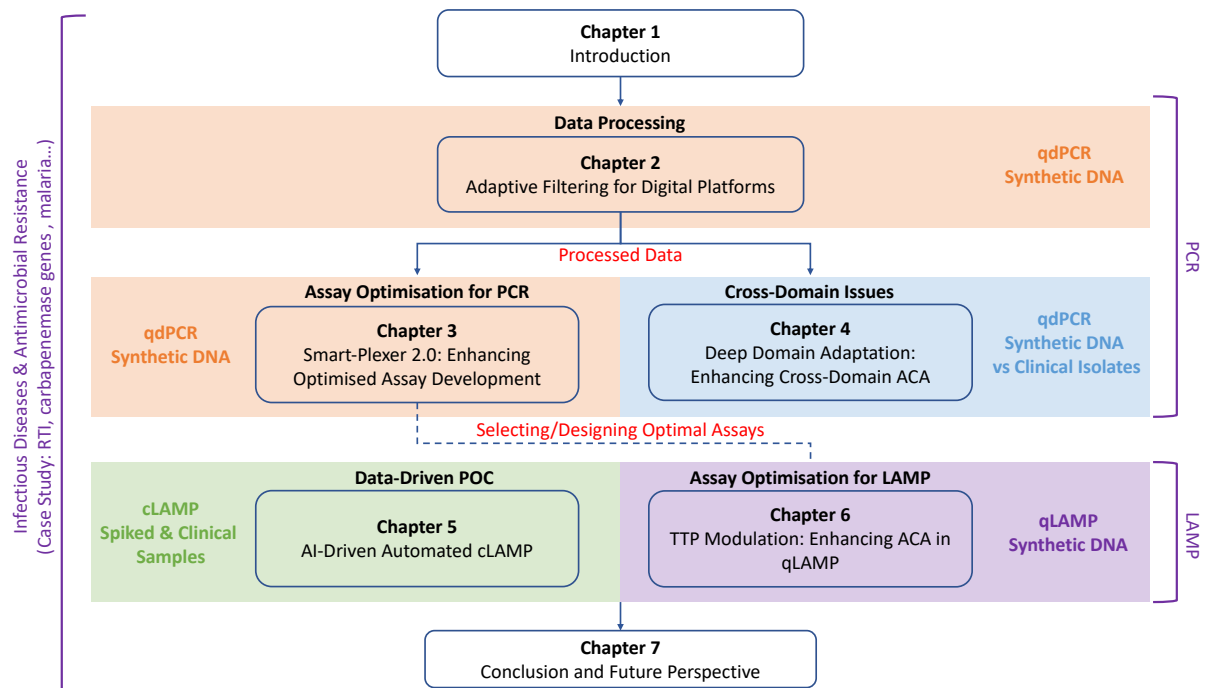


Figure 1.6: Thesis structure.

1.5.2 Materials and Data

The data source overview of the thesis is also depicted in Figure 1.6. While Chapter 2 and Chapter 3 utilise qdPCR data generated by synthetic DNA for developing the methodologies, Chapter 4 introduces both synthetic DNA and real clinical isolates in validating the framework. In Chapter 5, hundreds of spiked and real clinical samples are involved in the evaluation of the algorithms. Chapter 6 uses synthetic DNA running in qLAMP to develop the TTP modulation. A variety of target DNA/RNA fragments linked to infectious diseases and AMR are involved in this thesis, including case studies in Respiratory Tract Infection (RTI), carbapenemase genes, malaria, among others.

All the qdPCR data utilised in this thesis are generated with Biomark HD/Biomark (Fluidigm Corporation, South San Francisco, California, United States) using the qdPCR 37KTM integrated fluidic circuit (IFC) provided by the same company. The 37KTM IFC is a microwell array system containing *nL*-level chambers moulded in PDMS. Detailed data descriptions can be found in the data section of each chapter.

1.5.3 Contributions of Research Chapters

Chapter 2 Adaptive Filtering for Digital Platforms: This chapter presents a framework of outlier detection on amplification curves to filter non-specific or low-efficiency reactions in dPCR data. Applied to the screening of carbapenemase genes, the Adaptive Mapping Filter improved classification sensitivity and reduced errors, demonstrating the value of kinetic-based filtering for enhancing assay performance and guiding further research.

Chapter 3 Smart-Plexer 2.0: Enhancing Optimised Assay Development: This chapter introduces Smart-Plexer 2.0, an enhanced framework for robust multiplex PCR assay selection. By incorporating novel kinetic features and clustering-based distance metrics, it improves classification accuracy and reduces variability of selected optimal assays, demonstrating reliable performance across complex experimental conditions and enabling scalable assay development on real-time PCR platforms.

Chapter 4 Deep Domain Adaptation: Enhancing Cross-Domain ACA: This chapter presents a transformer-based conditional domain adversarial network (T-CDAN), which is a transformer-based domain adaptation model transferring from synthetic DNA to clinical dPCR data for improved pathogen identification. Tested on 198 clinical isolates, T-CDAN achieved 93.1% curve-level and 97.0% sample-level accuracy, significantly outperforming prior methods and enabling high-level multiplexing without hardware changes, also providing a promising way to eliminate domain discrepancies of amplification data for further clinical scenarios.

Chapter 5 AI-Driven Automated Colorimetric LAMP: This chapter presents an AI-driven workflow for automated cLAMP detection using tube-strip photos and deep learning. Evaluated on 1,636 images from 527 clinical tests across three diagnostic panels, it achieved over 96% invalid rejection and over 99% colour detection accuracy, demonstrating robust, scalable performance for point-of-care, cloud-integrated diagnostics.

Chapter 6 Time-to-Positive Modulation: Enhancing ACA in qLAMP: This chapter presents a strategy to modulate LAMP amplification curves by adjusting primer concentrations, enabling target separation via TTP shifts. A power model and global optimisation minimise overlap, while binary ACA resolves ambiguities. This hybrid framework enhances multiplex qLAMP accuracy, offering scalable, automated assay design for diagnostics.

Chapter 2 Adaptive Filtering for Digital Platforms

2.1 Chapter Overview

As discussed in 1.3.1, when adapting ACA in digital platforms for the development of machine learning algorithms, accurate target classification is compromised by the presence of undesired amplification events and non-ideal reaction conditions. Therefore, this chapter proposes a novel framework to identify and filter out non-specific and low-efficiency reactions from real-time PCR data using outlier detection algorithms based solely on the sigmoidal trends of amplification curves. As a proof-of-concept, this framework is implemented to improve the classification performance of screening carbapenemase-producing organisms in clinical isolates. A novel strategy, named Adaptive Mapping Filter (AMF), is proposed to adjust the percentage of outliers removed according to the number of positive counts in qdPCR. From an overall total of 152,000 amplification events, 116,222 positive amplification reactions were evaluated before and after filtering by comparing against the melting peak distribution, proving that abnormal amplification curves (outliers) are linked to shifted melting distribution or decreased PCR efficiency. ACA was applied to assess classification performance before and after AMF, showing an improved sensitivity of 1.2% when using inliers compared to a decrement of 19.6% when using outliers (p -value < 0.0001), removing 53.5% of all wrong melting curves based only on the amplification shape. This chapter explores the correlation between the kinetics of amplification curves and the thermodynamics of melting curves, and it demonstrates that filtering out non-specific or low-efficiency reactions can significantly improve the classification accuracy for ACA, paving the way for the following chapters on applying advanced assay design selection and target identification approaches.

The concept and experimental work presented in this chapter resulted in the following publication:

L. Miglietta*, K. Xu*, P. Chhaya, L. Kreitmann, K. Hill-Cawthorne, F. Bolt, A. Holmes, P. Georgiou, J. Rodriguez-Manzano, ‘Adaptive Filtering Framework to Remove Nonspecific and Low-Efficiency Reactions in Multiplex Digital PCR Based on Sigmoidal Trends’, *Analytical Chemistry*, vol. 94, no. 41, pp. 14159–14168, Oct. 2022.

***Joint first authorship, contribution:** conceptualisation, methodology, software, validation, formal analysis, investigation, data curation, writing-original draft, writing-review & editing, visualisation. I served as the primary researcher for this study, independently handling all data science-related work, including but not limited to algorithm development, coding, data validation, and result analysis.

Some figures and tables in this chapter and in the corresponding appendix are reproduced or adapted from the abovementioned publication, and are reused with permission from the publisher under the author rights policy: CC BY 4.0 [43].

2.2 Introduction

This chapter demonstrates that undesired amplification reactions from qdPCR can be detected and filtered out by evaluating only the sigmoidal shape of the amplification curve. Here, a novel methodology is proposed that can be used with multiplex PCR assays without the need for post-amplification analysis, increasing the accuracy and reliability of the results [112], [113].

During the last decade, gold-standard PCR technologies, along with other nucleic acid amplification chemistries, have resulted in key procedures for molecular diagnostics in both academic and clinical environments [114], [115]. However, limitations such as sample availability, trained personnel, and overall laboratory costs can represent obstacles to the scalability and adoption of PCR-based approaches [116], [117]. To overcome these barriers, multiplexing has been used to unlock the potential of conventional instruments, increasing the number of targets that can be detected in a single reaction [13], [84]. Since the adoption of multiplexing techniques, researchers and industries have successfully applied them to different areas such as molecular diagnostics, RNA signature polymorphism, and quantitative analysis [118], [119], [120]. Moreover, in an effort to increase overall multiplex PCR capabilities, numerous studies have recently been published on the use of ML to identify the biological nature of an amplification event, improving throughput, clinical and analytical reliability, and sample classification accuracy [85], [121]. While data-driven methods have mostly been employed to improve the accuracy of target identification, with the aim of increasing multiplexing capability, some groups have also explored such techniques for outlier removal, both in digital and bulk PCR. For instance, Yao et al. [86] developed a process-based classification model to identify false positive curves in qdPCR (leading to a 64% improvement compared with classical techniques), and Burdukiewicz et al. [122] developed an algorithm to

automatically detect hook effect-like curvatures, allowing for streamlined quality control in qPCR.

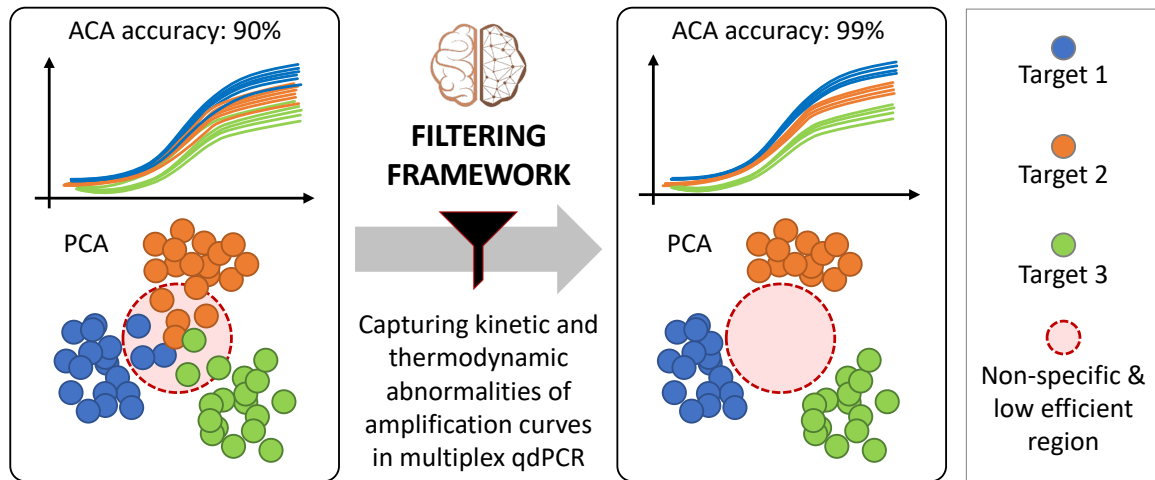


Figure 2.1: Concept figure of the proposed qdPCR filtering. Left: Raw amplification curves and their corresponding ACA clusters (represented by principal component analysis) include non-specific and low-efficiency reactions (confined within the red-circled region). The presence of outliers blurs the boundaries of the different clusters, negatively impacting ACA classification accuracy. By applying the proposed filtering framework, kinetic and thermodynamic abnormalities from amplification events can be captured. Right: Outliers are removed from the original data, resulting in more separated clusters and clearer boundaries. Therefore, the ACA classification accuracy is improved.

A barrier to the broader adoption of DDM techniques is that they may be limited by instrumentation specifications, such as thermal profile performance, available optical channels and filters, and software setup. Across all these ML-based multiplexing strategies, the ACA approach can be negatively affected by the presence of abnormal amplification products, due to primer dimerisation, amplification of undesired targets, the miscalibration of the instrument, and intra-molecular secondary structures. These abnormal behaviours tend to alter the kinetic information of the sigmoidal curves, resulting in low efficiency or delaying the amplification reaction [96], [123], [124]. As represented in Figure 2.1, when considering shapes of amplification curves from a multiplex assay, similarities among different targets can reduce the accuracy of the ACA classifier, as the presence of non-specific or low-efficiency reactions results in blurred boundaries among clusters. To overcome this problem, an intelligent algorithm will be developed in this chapter to filter out outliers from multiplex amplification events. Furthermore, to validate the correctness of outlier removal, amplification curves (which can be divided into *inliers* and *outliers*) are compared with labelled melting curves (which can be categorised into *correct* and *wrong*). Detailed data descriptions are provided later in 2.3.1.

In this chapter, it will be shown that non-specific and low-efficiency PCR reactions affect the shape of the amplification curve; therefore, they can be filtered out by considering only the sigmoidal trend. Furthermore, an outlier removal algorithm called Adaptive Mapping Filter (AMF) is developed, which, in combination with the ACA approach, is used to improve the multi-target classification accuracy. This represents a step forward in incorporating the ACA in clinical applications and ensuring that, by filtering in the correct amplification curves, higher diagnosis reliability is delivered to the patient. These concepts are explored using data obtained from qdPCR experiments reported by Miglietta et al, 2021 [69]. As a case study, three of the “The big 5” carbapenemase genes (*bla_{NDM}*, *bla_{IMP}*, and *bla_{OXA-48}*) were considered in this study.

By sharing this new approach, we can significantly improve the quality of data from qdPCR instruments and enhance the sensitivity and accuracy of ML-based multiplexing methods relying only on amplification curves. Moreover, extending this framework to other amplification chemistries and real-time platforms will improve the multiplexing capabilities of existing diagnostic workflows and platforms.

2.3 Experimental Section

In this section, a new framework for outlier removal in qdPCR is proposed. As depicted in Figure 2.2, this framework took raw amplification curve data as input, and applied baseline and flat/late curve removal in the processing step. Then each processed curve was fitted by a sigmoid function, and the fitted parameters, as well as a newly developed feature referred to as S_{end} , were used as input for a filtering algorithm which identified outliers automatically. Finally, the framework outputs the amplification curves after filtering, marked as inliers.

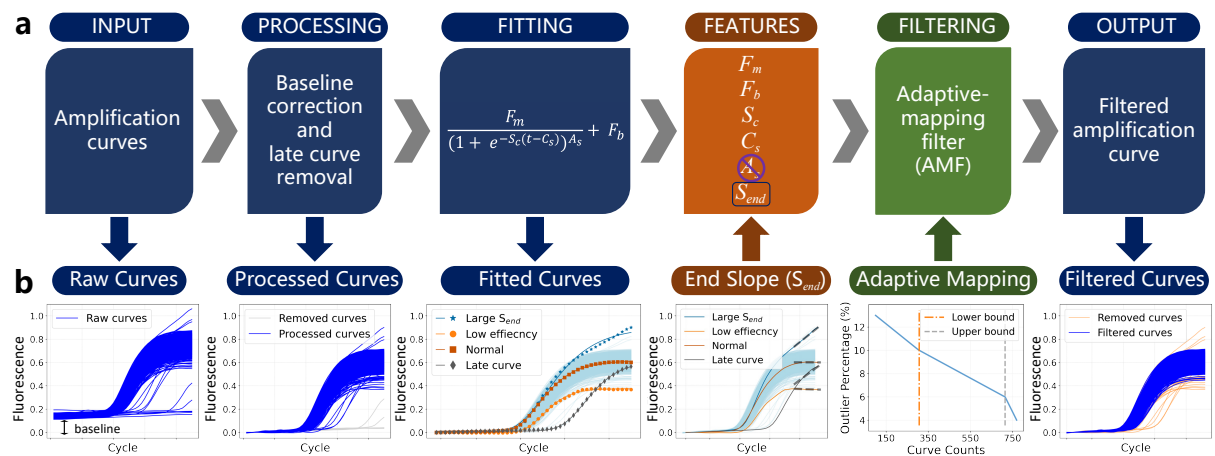


Figure 2.2: Detailed framework of the proposed qdPCR filtering. a) Framework steps: raw data input, processing, curve fitting, feature extraction, AMF and filtered curve output. b) Input or

output of each step. From left to right, the inputs of the framework were raw amplification curves, some of which are flat or late curves. By applying the processing step, the baselines were removed, and flat/late curves were discarded. Following this, the processed curves were fitted using a five-parameter sigmoid function, after which each curve was condensed into five features. A new feature S_{end} , which indicates the ending slope of the AC, plus four of the parameters from the five-parameter sigmoidal fitting, were used to form a set, which is the input of the filtering step. The A_s parameter was discarded from the feature set as it is unsuitable for the adopted algorithms. We further developed the AMF with a monotonic decreasing map between positive curve numbers within a panel and the outlier percentage. The outputs of the framework are the curves after filtering (inliers).

2.3.1 Data Input

As a case study, data from Miglietta et al. 2021 were used in this work [69]. Data from synthetic DNA (gBlocksTM gene fragments, IDT) containing *bla*_{NDM} (N=18,480), *bla*_{IMP} (N=17,710), and *bla*_{OXA-48} (N=17,710) gene sequences were used as the standard clean dataset. These three targets were selected because of their high prevalence and clinical significance in UK hospitals. From the original study, a total of 198 clinical isolates labelled with these three targets were used as the testing samples in order to maintain a balanced dataset. Each sample contained 770 raw amplification curves for a total of 152,460 ACs across all the samples, within which 116,222 were positive after the processing step. It is expected that data from clinical isolates are much noisier and thus contain more outliers than those from gBlocks. The detailed assay design strategy is described in Section A.1.

2.3.2 Data Processing

The first step of the framework is processing the raw curves using a baseline correction and a flat/late curve removal to exclude the negative curves of the unprocessed data from the qdPCR output. The baseline of the real-time PCR reaction during the initial cycles presents little change in fluorescent signal. The low-level signal of the baseline equates with the background fluorescence or noise of the reaction. Therefore, the baseline of each raw curve was processed by averaging the fluorescent value of the first five cycles and subtracting it from the time series. Following this, flat/late curves were removed by applying an upper and lower fluorescence threshold at the 40th cycle, as suggested by the manufacturer [68], [125].

2.3.3 Fitting and Feature Extraction

Following the processing step, a curve fitting step was introduced to represent the processed amplification curves with sigmoid parameters, which were later down-selected and used as

input features for outlier removal and classification algorithms. A five-parameter sigmoid model [124], which is shown below, was used to fit the amplification curves:

$$F(t) = \frac{F_m}{(1 + e^{-S_c(t - C_s)})^{A_s}} + F_b, \quad (2.1)$$

where t is the PCR cycle number, $F(t)$ is the fluorescence at the t^{th} cycle, F_b is the background fluorescence, F_m is the maximum fluorescence, S_c relates to the slope of the curve, C_s is the fractional cycle of the inflection point, and A_s is the asymmetric parameter. To solve the non-linear least-squares optimisation problem for the curve fitting, the Trust Region Reflective (TRF) algorithm with specific bounds was used [126]. Here, the upper and lower bounds were set as $[10, 0.3, 10, 50, 100]$ and $[0, -0.1, -10, -50, -10]$ for the search of the five-parameter set $p = [F_m, F_b, S_c, C_s, A_s]$. The initial parameter set p_0 was optimised through pivot fitting on 5% of the training data. After fitting, each amplification curve was given as five parameters, which are condensed representations of curve information. The fitting quality was assessed using Mean Squared Error (MSE) and reported in Figure A.1. All parameters except for A_s were considered as input features for outlier removal algorithms because parameter values of outliers may have significant differences from those of normal curves. The A_s parameter shows a bimodal distribution with two distant peaks, which is unsuitable for the outlier removal step because many of the outlier algorithms require a unimodal distribution of features. Therefore, it was discarded from the feature set for filtering.

In addition, a new feature called the end slope (S_{end}) was introduced with the aim to provide further information about the amplification curve shape. This was calculated by taking the average of the first derivatives at the last five cycles of the amplification curve:

$$S_{end} = \frac{1}{5} [D(N-4) \quad D(N-3) \quad \cdots \quad D(N)] \mathbf{e}_5^T, \quad (2.2)$$

where

$$D(x) = \frac{dF(t)}{dt}, \quad (2.3)$$

$$\mathbf{e}_5 = [1 \quad 1 \quad 1 \quad 1 \quad 1], \quad (2.4)$$

and N is the total cycle number.

Using the S_{end} feature, the information in the tail of amplification curves was extracted, which contributes to distinguishing inliers and outliers. For example, as illustrated in the ‘‘Fitting Curves’’ step of Figure 2.2(b), curves that do not reach the plateau may have larger end

slopes. These curves cannot be precisely represented by the fitted parameters since the fitting equation is not capable of capturing this non-plateaued trend. Therefore, S_{end} will benefit the result of outlier removal by providing additional information to the feature set. Including S_{end} and discarding A_s , the final feature set for outlier removal algorithms is $\mathbf{x}_f = [F_m, F_b, S_c, C_s, S_{end}]$.

2.3.4 Outlier Removal Algorithms

In this chapter, seven outlier removal algorithms were considered, which can be split into the following categories according to their principal ideas of filtering: proximity-based, linear, outlier ensembles, and angle-based algorithms. (1) Proximity-based outlier detection algorithms rely on using a distance metric (e.g., Euclidean or Manhattan) to identify outliers. We applied two proximity-based algorithms, which are Local Outlier Factor (LOF) [127] and Density-based Spatial Clustering of Applications with Noise (DBSCAN) [128]. The LOF algorithm considers the k -nearest neighbors (KNN) to every point in the dataset and computes a local outlier factor for each of them. DBSCAN classifies the points into the core, border, and noise of clusters based on the number of points within the radius of the considered point. (2) The linear outlier detection methods used were One-Class Support Vector Machine (OC-SVM) [129] and Elliptical Envelope [130]. OC-SVM applies the concept of finding a hyperplane that separates the inlier points from the origin, such that the hyperplane is as close as possible to the inlier points. The Elliptical Envelope aims to fit the smallest ellipse possible to the core cluster of data points, with any point outside being considered an outlier. (3) Outlier ensemble-based detection methods considered were Isolation Forest [131] and Feature Bagging [132]. Isolation Forest uses random forests to recursively randomly partition data, after which data points with fewer partitions to isolate are marked as outliers. Feature bagging considers multiple outlier algorithms and randomly selects a group of features. From those features, the resulting outlier scores from each algorithm are merged to find the strongest outliers. (4) Angle-based Outlier Detection considers the angles made by a point with all other pairs of points in the dataset [133]. For each point, the variance is calculated from all the angles obtained. For a potential outlier, the variance is small because the point is distant from the main cluster of data.

2.3.5 Adaptive Mapping Filter (AMF)

Most of the outlier detectors explained in the previous section require a hyperparameter called “contamination ratio” or “outlier percentage”, which represents the percentage of outliers to be

removed from the original data. To adaptively set up this hyperparameter, a mapping strategy was developed, mapping the number of positive reactions per panel in the qdPCR chip (processed curves) to the contamination ratio used in the outlier removal algorithm.

In digital PCR, as the number of positive curves increases, the probability of having more than one molecule in a single well increases, resulting in a shift of reaction state from digital to bulk. Moreover, as the reaction goes toward the bulk region, a higher number of positive curves will present in a panel, which can result in a lower probability of observing a non-specific or low-efficiency reaction (outlier) in a well [19], [134]. Let us suppose that for each well, the probability of observing an outlier is $p(M_i)$, where M_i is the number of processed curves for the i^{th} sample. Since $p(M_i)$ are independent and identically distributed (*i. i. d.*) for all the wells, the total number of outliers X_i observed in the i^{th} sample follows the distribution of $X_i \sim B(M_i, p(M_i))$. Therefore, the expected percentage of outliers in the i^{th} sample should be:

$$\text{outlier percentage} = \frac{E(X_i)}{M_i} = \frac{M_i p(M_i)}{M_i} = p(M_i), \quad (2.5)$$

which means that the expected outlier percentage is a monotonic decreasing function to the number of positive curves. In this chapter, a piecewise linear function with empirical turning points was applied, as illustrated in the filtering step of Figure 2.2(b).

Coupling the adaptive mapping with an outlier removal algorithm, a novel method called Adaptive Mapping Filter (AMF) was developed, which takes the feature set as input and outputs the inliers.

2.3.6 Melting Labelling

An algorithm was developed to automatically label the melting curves as specific (which we called “correct”) or non-specific (referred to as “wrong”) ones. By using this methodology, the percentage of wrong melting curves within all the curves of a sample (Wrong Melting Percentage or WMP) was calculated, and this WMP further served as a metric for performance evaluation.

To apply melting labelling, the reference melting peak for each target needs to be determined. For a target $tg \in [bla_{\text{NDM}}, bla_{\text{IMP}}, bla_{\text{OXA-48}}]$, a reference melting peak temperature T_m^{tg} was given by calculating the median value of all the melting peak temperatures of the gBlock curves with Target tg . After that, the steps below were followed to label every single melting curve of the clinical dataset:

- (1) Find the global maximum melting peak’s temperature T_m^g of the current melting curve.

- (2) If $T_m^g \notin [T_m^{tg} - \frac{W}{2}, T_m^{tg} + \frac{W}{2}]$, where W is the tolerance width of the T_m^g distribution, the current curve is labelled directly as a wrong melting curve. Here, considering our instrument resolution for melting curve analysis, our W is equal to ± 0.5 °C.
- (3) Otherwise, find the local maximum melting peaks' temperatures on the left and right sides of T_m^g on the current curve, mark them as T_m^l and T_m^r , respectively. Note that either T_m^l or T_m^r may not exist. If neither exists, the current curve will be labelled as a correct melting curve.
- (4) If at least one of T_m^l and T_m^r exists, a set of this (these) local melting peak(s) will be constructed. For each element T_m^e in this set, check whether

$$H_e \in [H_{mean} - 4H_{std}, H_{mean} + 4H_{std}], \quad (2.6)$$

where H_e is the height of the current melting curve at temperature T_m^e , H_{mean} and H_{std} are the mean and standard deviation of $[H_{1,T_m^e} \ H_{2,T_m^e} \ \dots \ H_{M,T_m^e}]$, in which H_{n,T_m^e} means the height of the n^{th} melting curve of the sample at temperature T_m^e , and M is the total curve number in the sample. If at least one of the above tests fails, the current curve will be labelled as a wrong melting curve. Otherwise, it will be marked as a correct one.

With the above steps, it is ensured that both curves with large deviations of T_m^g from reference melting peaks and curves with large non-specific local melting peaks can be labelled as wrong. In this way, all the curves had been marked as either “correct” or “wrong”, and further used to calculate the WMP:

$$\text{WMP} = \frac{N_{wrong}}{N_{total}} \times 100, \quad (2.7)$$

where N_{wrong} is the number of wrong melting curves within the sample, and N_{total} is the total number of curves in the sample.

It is worth mentioning that the proposed algorithm of automatic melting labelling is not a part of the filtering framework. The labelling was used to calculate the WMP, which functioned as a metric for filtering evaluation, where a lower WMP indicates better filtering performance.

2.3.7 Data Visualisation

Visualisation is a vital step for understanding the distribution of a given dataset. In this chapter, PCA with two components was used to visualise the feature sets of the curves before and after applying the outlier removal algorithm in scatter plots. Visual inspection was performed to

illustrate how separated the clusters of different targets were. Following this, metrics for measuring density and degree of separation among those clusters were used to quantitatively evaluate how well they were divided.

Specifically, after the PCA of the feature set $\mathbf{x}_f = [F_m, F_b, S_c, C_s, S_{end}]$ from the amplification curves of each target, the Silhouette Coefficient for each feature set was calculated [135]. The mean value of these coefficients, known as the mean Silhouette Score, was then used to indicate how well the curves of the same targets are clustered. A higher mean Silhouette Score implies denser and better-separated clusters are observed. Two additional metrics, the Calinski-Harabasz score [136] and the Davies-Bouldin score [137], were also implemented for clustering evaluation, where a higher Calinski-Harabasz score or a lower Davies-Bouldin score relates to larger inter-cluster distances among targets.

2.3.8 Classification of Amplification Curves based on DDM

The ACA method uses kinetic information encoded in the amplification curve to classify different nucleic acid molecules from a PCR test. The performance of the ACA was assessed using different curve representations (Table A.1), and the five fitted parameters were used in this work. To illustrate the influence of the AMF on the ACA, a random forest classifier with 100 trees was applied to the feature set $\mathbf{x}_c = [F_m, F_b, S_c, C_s, A_s]$, which differs from the $\mathbf{x}_f$ used for outlier removal algorithms. Here, the parameter A_s was reintroduced because more curve-related information is needed, provided that the proposed classifier is relatively less sensitive to the feature distributions. S_{end} was discarded for classification because, after outlier removal, abnormal curves with large end slopes were not present in the dataset. For the remaining curves, S_{end} were close to zero, thus it was not necessary for S_{end} to be included again. All the other features were normalised with the mean and the variance of the training data before being input into the classifier.

In this chapter, after applying data processing and feature extraction on both the training and testing sets, the extracted features of the training set were used to train a Random Forest classifier. This trained classifier was then evaluated on the testing set with or without Adaptive Mapping Filter (the progress of AMF is totally unsupervised, so it can be applied on the testing dataset without true labels). For the testing set, we utilised both the inliers and the outliers marked by the AMF algorithm and tested them respectively. As a comparison, two randomly down-selected datasets with the same numbers of curves as the inliers and the outliers were also constructed and tested.

2.3.9 Statistical Analysis

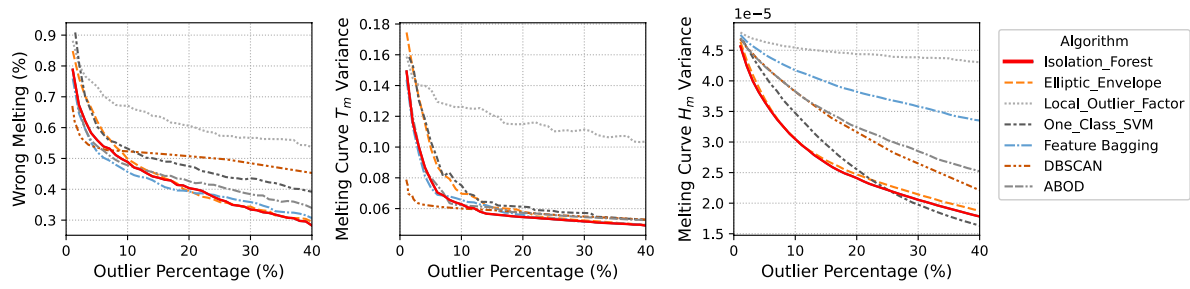
Two-sided Wilcoxon signed-rank tests were used to determine the statistical significance of the changes in WMP and melting peak distributions (distributions of melting peak temperature, T_m , and height, H_m) before and after outlier removal. Two-sided Mann-Whitney U rank tests were used to compare the distributions of C_t , FFI, and maximum slopes between inlier and outlier amplification curves. These three metrics were chosen for their relationship with the amplification curve efficiency. Many studies suggest that sigmoidal modelling of the entire amplification curve can be used to define the rate of PCR efficiency. Therefore, low-efficiency PCR reactions are related to low fluorescent values and low maximum slope [100], [138].

Moreover, the significance of the comparison between inliers and outliers in clustering Silhouette coefficients was determined by a two-sided Wilcoxon signed-rank test. This test was also used in the evaluation of the classification performance. A p-value of 0.001 with Bonferroni correction was used as the threshold for statistical significance.

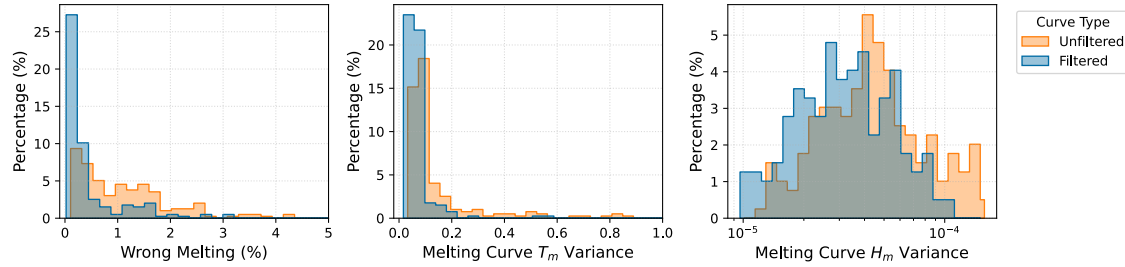
2.4 Results and Discussion

In this chapter, a new framework is presented to detect outliers from amplification reaction in qdPCR. The outlier identification relies on the AMF, which is comprised of an outlier detection algorithm and a mapping strategy to adapt the contamination ratio hyperparameter to the positive amplification reaction counts (or positive wells) of the qdPCR chip.

a) Melting performance metrics vs outlier percentage using 7 filtering algorithms



b) Distribution of melting performance metrics using Isolation Forest

c) Example of a *bla*_{OXA-48} clinical isolate before and after Adaptive Mapping Filter (AMF)

Wrong melting percentage: 3.51% (before AMF) - 1.08% (after AMF)

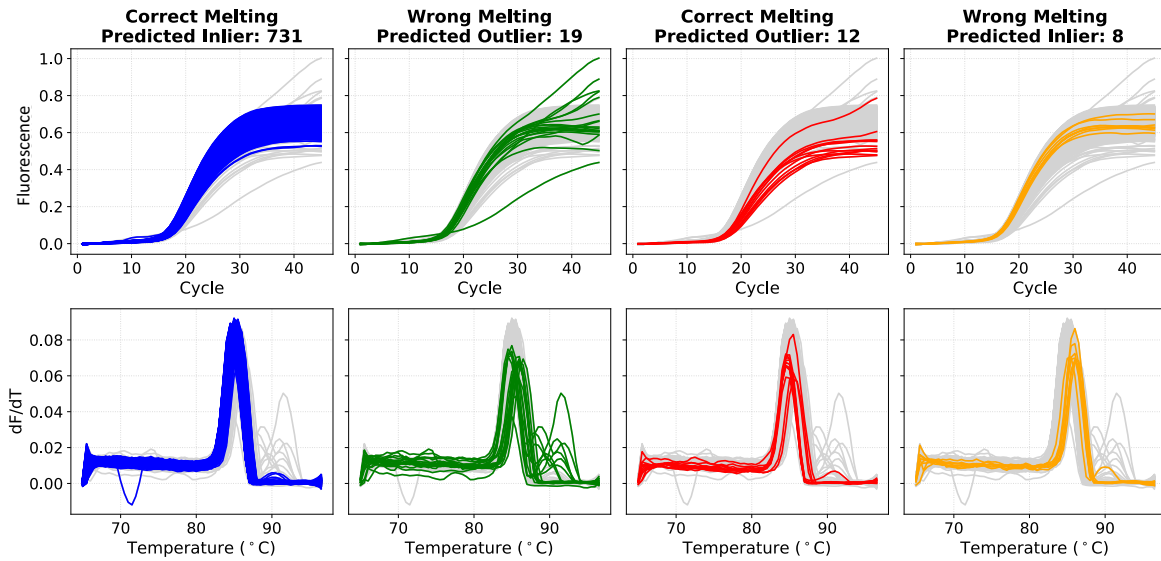


Figure 2.3: Melting curve analysis on filtering results. a) Melting performance shown with WMP, T_m and H_m variances versus fixed outlier percentage. As the outlier percentage increases, all the metrics show decreasing trends, which tend to plateau after a certain percentage. As illustrated by the firm red line, Isolation Forest performs the best overall for the three metrics. b) The distribution of melting performance metrics shows that, after filtering, the WMP becomes significantly smaller, and T_m and H_m have a narrower distribution. c) An example of *bla*_{OXA-48} clinical isolate. Each column shows the amplification curve and corresponding melting curve of the correct melting and predicted inliers (N=731), wrong melting and predicted outliers (N=19), correct melting and predicted outliers (N=12), wrong melting and predicted inliers (N=8).

2.4.1 Evaluation of Outlier Detection Algorithms

As shown in Figure 2.3(a), we evaluated the detection performance of seven outlier removal algorithms on filtering amplification curves against outlier percentages by using three metrics: (I) WMP, (ii) Melting Curve T_m variance, and (iii) Melting Curve H_m variance. The changing values of metrics for different algorithms with fixed outlier percentages from 0.1% to 40% are shown in Figure 2.3(a). After the filtering is applied, the WMP shows a significant reduction from 1.1% (from the unfiltered dataset) to a maximum of 0.9% after filtering across all the algorithms. The graph depicts that the outlier percentage and WMP are inversely proportional, but the trend can vary among methods. Proximity-based outlier detectors perform worse overall compared to the rest, so they are unable to achieve a dramatic decrease in WMP, even with large contamination ratios. On the other hand, ensemble-based detectors such as Feature Bagging and Isolation Forest have better performance with the lowest WMP among all the outlier percentages. As shown in the centre and right end graphs, the variances of T_m and H_m have a decreasing trend as the outlier percentage increases, indicating that both of their distributions are narrowed down. In the T_m variance plot, it is noticed that DBSCAN achieves better performance at lower outlier percentages, but this trend reaches a plateau as the outlier percentage further increases. Once again, ensemble-based methods have similar behaviour for the T_m variance as for the WMP. For instance, Isolation Forest outperforms all other detectors after the outlier percentage reaches 12%. Moreover, Isolation Forest and Elliptic Envelop show the best performance for H_m variance up to a 26% contamination ratio.

In this analysis, WMP was used to show the change of wrong melting proportion after applying outlier detection algorithms, indicating the direct effect of the filtering on removing wrong melting curves. It is important to consider that we do not relate wrong melting with wrong target sequences, as the true nature of the amplicons resulting from the PCR reaction can only be established by sequencing, which is impractical in digital PCR. The WMP is used to evaluate the shift of the melting peak or the presence of multiple low-intensity peaks, which result from non-specific or low-efficiency amplification reactions. This can largely affect the ACA classification depending on the presence of the abovementioned phenomena; therefore, filtering out such events can result in improved target identification. Moreover, a smaller T_m variance indicates a narrower T_m distribution, which, in combination with the WMP methods, shows that curves with large deviations from the reference T_m^{tg} are removed by the filtering algorithm. In molecular biology, those curves may be generated after non-specific events such as undesired target interaction or primer dimerisation [139]. In addition, melting curves

presenting low $-df/dt$ (or H_m) are associated with low-efficiency amplification reactions. Therefore, narrowed distribution of H_m indicates that low-efficiency curves, which are present at the tail of the distributions, are removed [140]. All the algorithms provide better performance compared to the original benchmark calculated on the unfiltered data. However, it is noticed that Isolation Forest is always among one of the best methods for all the metrics and does not show any defects that are for other algorithms (outlier distribution reported in Figure A.2). In the following sections, we used Isolation Forest to further demonstrate the proposed framework.

2.4.2 Filtering Performance Analysis of AMF

In the following step, AMF was applied to the unfiltered data, and the distributions of inner-sample WMP, T_m and H_m variances are illustrated in Figure 2.3(b). Across these three metrics, significant shifts of distributions to smaller values are shown after filtering, supported by all the p -values < 0.0001 . This indicates that the proposed AMF can significantly remove both non-specific and low-efficiency curves only by looking at amplification curves. This proves our hypothesis that amplification curves contain not only kinetic but also thermodynamic information, as numbers of outliers correspond to wrong melting curves.

An example of the AMF visual performance on a clinical isolate containing the carbapenemase gene *bla_{OXA-48}* is illustrated in Figure 2.3(c). Columns represent both amplification and melting curves of: (I) correct melting and predicated inliers (N=731, 94.9%), (ii) wrong melting and predicted outliers (N=19, 2.5%), (iii) correct melting and predicted outliers (N=12, 1.6%), and (iv) wrong melting and predicted inliers (N=8, 1%). The first column shows the correctly identified inliers representing specific products of PCR tests. In the second column, non-specific reactions are correctly identified and labelled as outliers, which emphasises the effectiveness of the filtering. It is noticed that a small number of specific curves were predicted as outliers, as shown in the third column of Figure 2.3(c). This phenomenon does not deny the efficacy of the filter, as these “incorrectly” removed curves have: (I) significantly larger C_t values, (ii) significantly smaller FFI, and (iii) smaller values of maximum slope compared to the inliers. Across the entire clinical isolate dataset (N=116,222), compared to melting curve analysis, 115,535 were correctly predicted inliers and 791 were correctly predicted outliers. Furthermore, 5,861 were wrongly classified as outliers, whereas 687 were wrongly classified as inliers. Further statistical analyses on the entire dataset also endorse these significant differences between inliers and outliers for C_t , FFI and maximum slope values, as illustrated in Table 2.1. This indicates that AMF removes certain curves because they are of low amplification efficiencies, even though they have “correct” melting

peaks. Less than ten curves labelled as “wrong” melting may be predicted as inliers, as shown in the fourth column of Figure 2.3(c). This can be explained by the relatively low temperature resolution of the equipment, which results in mislabelled wrong melting curves due to the large quantisation noise of T_m^g during temperature measurement. In fact, by visually inspecting the last column of Figure 2.3(c), it can be seen that amplification curves are of similar shapes to correctly predicted inliers (shown in the first column of Figure 2.3(c)). The WMP of the illustrated sample has dropped from 3.51% to 1.08%. Overall speaking, in this demonstrated dataset, 1.2% of wrong meltings were reported before filtering, and after applying AMF, we reduced the WMP by half to 0.59%.

Table 2.1: Comparison of C_t , FFI, and maximum slope between predicted inliers and outliers with correct melting peaks.

Target	C_t (mean $\pm$ std)		FFI (mean $\pm$ std)		Max Slope (mean $\pm$ std)	
	Inliers	Outliers	Inliers	Outliers	Inliers	Outliers
<i>bla_{NDM}</i>	21.45 $\pm$ 3.28	26.40 $\pm$ 6.11	0.67 $\pm$ 0.06	0.60 $\pm$ 0.12	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01
<i>bla_{IMP}</i>	30.33 $\pm$ 2.23	31.25 $\pm$ 3.43	0.44 $\pm$ 0.06	0.41 $\pm$ 0.07	0.0276 $\pm$ 0.003	0.0271 $\pm$ 0.01
<i>bla_{OXA-48}</i>	18.82 $\pm$ 3.08	21.03 $\pm$ 4.34	0.65 $\pm$ 0.08	0.51 $\pm$ 0.16	0.05 $\pm$ 0.01	0.04 $\pm$ 0.02

For all the targets, inliers have significantly smaller C_t and larger FFI and max slope, with all p -values < 0.0001 .

2.4.3 Feature Set Visualisation

To visualise the effect of the AMF, PCA-based feature visualisation before and after filtering is depicted in Figure 2.4. On the left of the figure, the unfiltered data shows larger overlapping within clusters of different targets and a higher number of outliers compared to the data after filtering. The segmented squares are used to emphasise the differences in cluster overlapping before and after the AMF, where clearer boundaries between *bla_{IMP}* and both *bla_{OXA-48}* and *bla_{NDM}* can be seen. These differences highlight that: (i) outliers can be effectively removed by the AMF, and (ii) removing outliers enhances the separation and reduces the overlap among different target clusters, which will ease the classification of the ACA method.

To numerically evaluate the degree of separation across target clusters, the mean Silhouette score of all the data points was calculated before and after filtering, showing an increment from 0.378 to 0.399 (p -value < 0.0001). In addition, the Calinski-Harabasz score increased from 101,002.729 to 130,134.802, and the Davies-Bouldin score dropped from 0.886 to 0.839. All those results indicate that AMF makes target clusters denser and better separated.

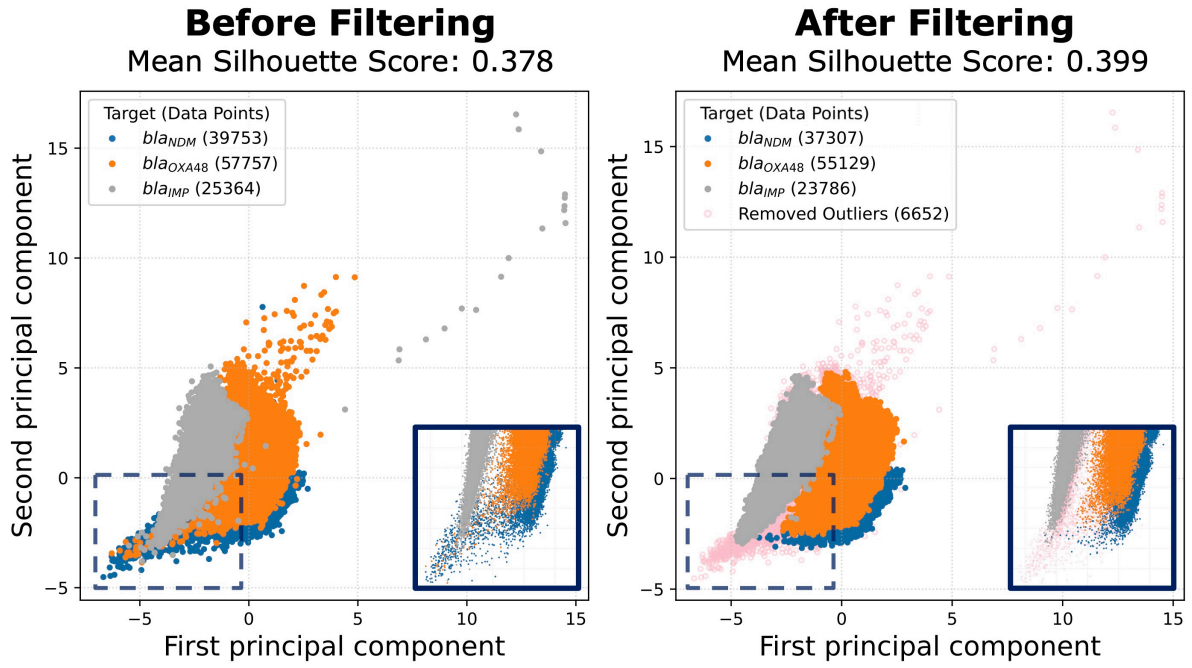


Figure 2.4: Data visualised using 2-D PCA before and after filtering. The processed data plot shows that most outliers have been removed from the original unfiltered data, and the clusters are more separated with clearer boundaries and fewer overlaps. The segmented squares on the bottom side of both figures show the areas where cluster overlapping is more evident, thus they are zoomed. The mean Silhouette Score rises from 0.378 to 0.399 after filtering.

2.4.4 ACA Classification

After demonstrating that removing outliers improves the overall distance among clusters, we further explored its impact on the ACA classification for both inliers and outliers against randomly down-selected datasets with the same number of curves. In Figure 2.5(a), the confusion matrix shows that the sensitivity for the inliers is 88.96%, which is an increase of 1.13% compared to the randomly down-selected ones (Figure 2.5(b)). For all the targets, a significant sensitivity improvement can be observed of 1.06%, 0.95%, and 1.39% for *bla_{IMP}*, *bla_{NDM}*, and *bla_{OXA-48}*, respectively. Moreover, the overall classification accuracy was 84.94% for inliers and 83.76% for randomly down-selected curves, showing a 1.18% improvement (p -value < 0.0001), which is in line with the overall WMP before filtering (WMP=1.2%). The presented improvement percentage is statistically significant, indicating that applying the filter will help increase the overall performance and specificity of the dataset. This supports our hypothesis that melting information or thermodynamics is contained in the amplification curve.

To show that the removed outliers are less informative for target recognition and harmful for the overall classification, in Figure 2.5(c)&(d) we show the confusion matrices of the classification using both removed outliers and a randomly down-selected dataset with the same

size. As expected, the performance for outliers is significantly worse than the randomly down-selected ones, with only 68.2% and 54.78% sensitivity and accuracy, respectively (p -values < 0.0001). This dramatic sensitivity decrement of 19.57% strongly suggests that outliers have less useful information for the classification of the selected targets.

Furthermore, the statistical analysis on the two randomly down-selected datasets shows no significant differences of in-sample accuracy with p -value = 0.448, which is in line with the central limit theorem as they originate from the same distribution. This is further proof that the efficacy of the proposed framework is not related to the size of the data.

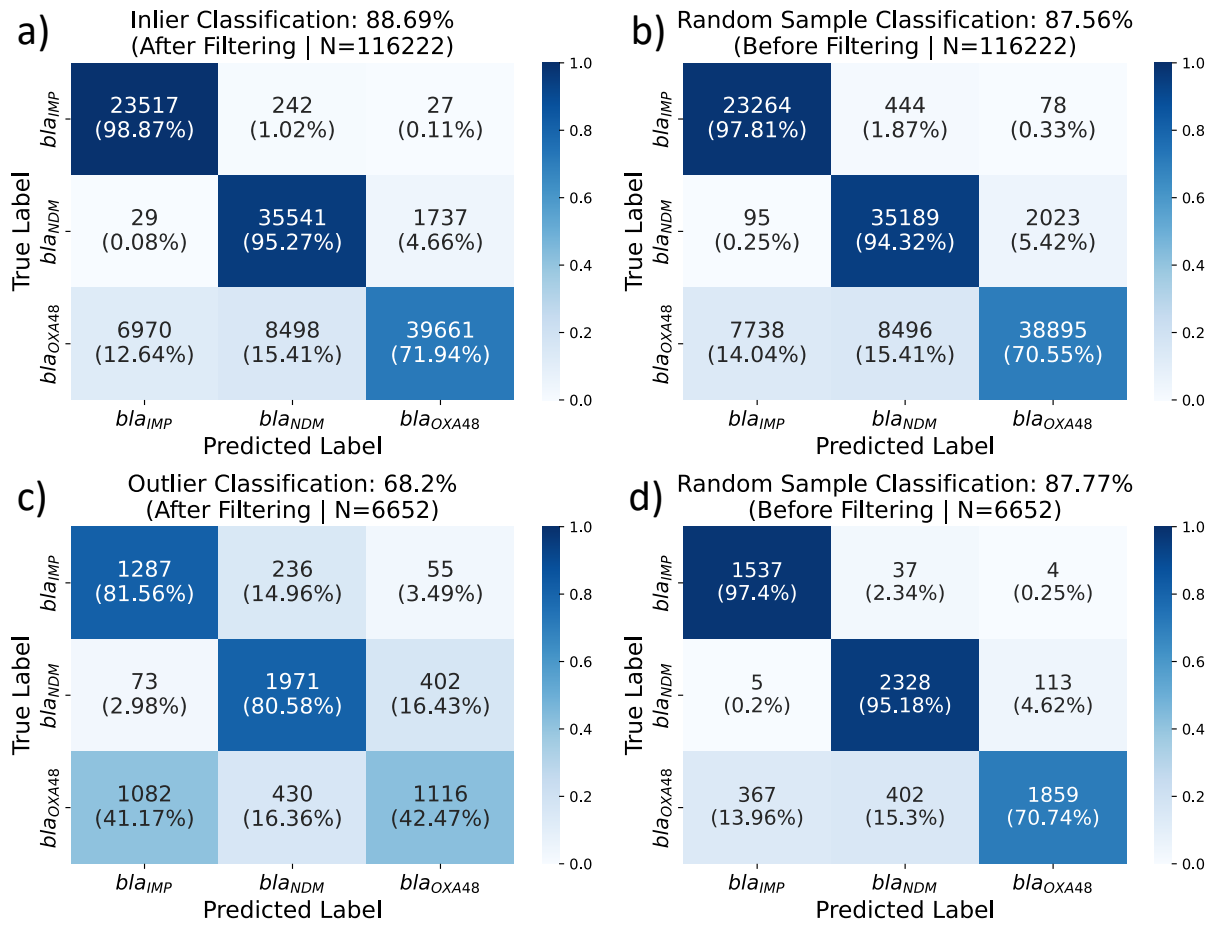


Figure 2.5: Confusion matrices for inlier and outlier classification. The four confusion matrices are shown for: a) inliers, b) randomly down-selected data with the same curve numbers as inliers, c) outliers, and d) randomly down-selected data with the same curve numbers as outliers. The title of each matrix reports the sensitivity of the model. Moreover, each square of the matrix has the number of predicted curves for the corresponding true label and the respective sensitivity of the square.

2.5 Conclusion

In this chapter, a novel framework to adaptively remove abnormal curves from real-time PCR amplification reactions was presented. The method takes the raw input from a qdPCR run and processes it in three steps: background subtraction, late curve removal, and sigmoidal fitting. Moreover, a new feature called end slope (S_{end}) is developed in this study, which, along with sigmoidal parameters, is used in the AMF. The AMF is capable of removing non-specific and low-efficiency amplification curves, which are labelled as outliers. Melting curves of the outliers, previously removed, were compared with melting curves of inliers using both WMP and melting peak distributions. Results show that non-specific and low-efficiency curves can be removed from the amplification reaction by purely considering the sigmoidal trend. Further validation of the framework's performance was conducted by assessing the classification accuracy and sensitivity of the ACA classifier on both inliers and outliers. This reinforces our hypothesis that removing abnormalities of amplification reaction in real-time PCR instruments would benefit data-driven multiplexing by removing undesired information.

In this chapter, data from qdPCR published by our group have been used to demonstrate the effectiveness of the proposed framework, but its generality has not been tested in other settings. Future work will focus on evaluating this methodology on real-time data originating from various qPCR instruments, from different chemistries (such as isothermal amplification), and from POC devices. qdPCR allows for generating amplification curves at low concentrations of samples, enabling the use of the developed framework. However, future work will focus on the application of this novel method in bulk reactions. Moreover, in the event of secondary amplification, the curve may show a second increasing phase with a large FFI and a deviated shape from sigmoid. However, as shown in Figure 2.2(b) fitting step, the approximate shape of the distorted curve can still be depicted by the five-parameter model, with still a relatively small fitting error. After fitting, certain parameter values of the secondary amplification events will be different and distant from normal reactions, and these events can be identified by the outlier detector. Regarding the presence of multiple targets in a single well, we expect to have a normal sigmoidal trend; therefore, the fitting error (MSE) will be low without affecting the AMF progress. However, the ACA classification of such event may be challenging. In our group's previous work, it was demonstrated that the presence of double targets can be resolved by using the AMCA approach [20], and other solutions such as FFI modulation by changing probe concentration in the TaqMan assay [18] may also help tackle this issue.

In conclusion, this chapter reveals the interconnection between the kinetics of the amplification curve and the thermodynamics of the melting curves. For the first time, a framework is introduced that is capable of removing abnormalities in kinetic and thermodynamic information by purely screening amplification curves. The methods proposed in this chapter pave the way for the research in the following chapters by providing a reliable unsupervised data pre-processing standard procedure.

Chapter 3 Smart-Plexer 2.0: Enhancing Optimised Assay Development

3.1 Chapter Overview

In the last chapter, an unsupervised approach was developed for eliminating non-specific and low-efficiency reactions from digital platform outputs, serving as the pre-processing for ACA. However, as mentioned in 1.3.2, to fully leverage the power of ACA in single-channel, single-well target identification, we need to make the amplification curves distinguishable. As part of this effort in computational-guided assay design, our group previously introduced Smart-Plexer 1.0, a tool that simulates multiplex assays using empirical singleplex data to identify optimal assay combinations *in silico*, maximising kinetic feature distances between targets to support ACA-based discrimination. While Smart-Plexer 1.0 performs reliably in controlled reactions and offers a strong framework for ACA assay design selection, it relies on a single kinetic feature and a median-base distance metric, which limits its accuracy in reactions with variable target concentrations or efficiencies. In this chapter, Smart-Plexer 2.0, a more robust and accurate version designed to improve performance in amplification reactions affected by such variability, is proposed. This version introduces three new kinetic features that are stable across different template concentrations, and uses clustering-based distance measures to better capture the variability between targets. Compared to its predecessor, Smart-Plexer 2.0 reduces accuracy variance by an order of magnitude and improves ACA classification by 1.5% and 1% in retrospective 3-plex and 7-plex assays, respectively. In a multi-experiment, cross-concentration evaluation of a newly developed 7-plex assay, it achieved 97.6% ACA accuracy, confirming its robustness across complex scenarios. Smart-Plexer 2.0 offers a reliable and scalable way to select high-performance multiplex PCR assays using standard real-time PCR instruments.

The concept and experimental work presented in this chapter resulted in the following published peer-reviewed article:

K. Xu*, L. Miglietta*, P. Kesakomol, A. Holmes, P. Georgiou, N. Moser, J. Rodriguez-Manzano, ‘Smart-Plexer 2.0: Leveraging New Features of Amplification Curves to Enhance the Selection of Multiplex PCR Assays in Multi-Target Identification’, *Analytical Chemistry*, vol. 97, no. 27, pp. 14311–14320, Jul. 2025.

***Joint first authorship, contribution:** conceptualisation, methodology, software, validation, formal analysis, investigation, resources, data curation, writing-original draft, writing-review & editing, visualisation. I served as the primary researcher for this study, independently handling all data science-related work, including but not limited to algorithm development, coding, data validation, and result analysis. I also conducted some parts of the wet-lab experiments.

Some figures and tables in this chapter and in the corresponding appendix are reproduced or adapted from the abovementioned publication, and are reused with permission from the publisher under the author rights policy: CC BY 4.0 [43].

3.2 Introduction

A primary challenge in the ACA is designing optimal assays capable of distinguishing multiple targets based on amplification curve shapes, particularly as the number of targets increases. Identifying optimal assays requires extensive experimental validation, with the testing burden growing exponentially as the number of targets increases. To address this challenge, our group previously introduced Smart-Plexer, a computational framework designed to streamline multiplex PCR assay development through in-silico optimisation of primer mixes [94]. As shown in Figure 3.1, with a number of candidate assays for each DNA target, this framework begins by experimentally applying singleplex assays and leveraging their amplification curves to simulate multiplex reactions, assuming that kinetic information will be maintained when transferring assays from singleplex to multiplex environments. Numerical kinetic features are then extracted from these ACs and statistically selected, representing the ACs in a reduced-dimensional space, and are used to calculate inter-target distances. Primer mixes with larger average (Average Distance Score, ADS) and minimal inter-target distances (Minimum Distance Score, MDS) are ranked higher and considered optimal for ACA performance. High-ranking primer mixes are tested empirically to determine the best one with the highest target classification accuracy. This simulation-driven approach significantly reduces extensive wet-lab testing, accelerating assay development while minimising costs.

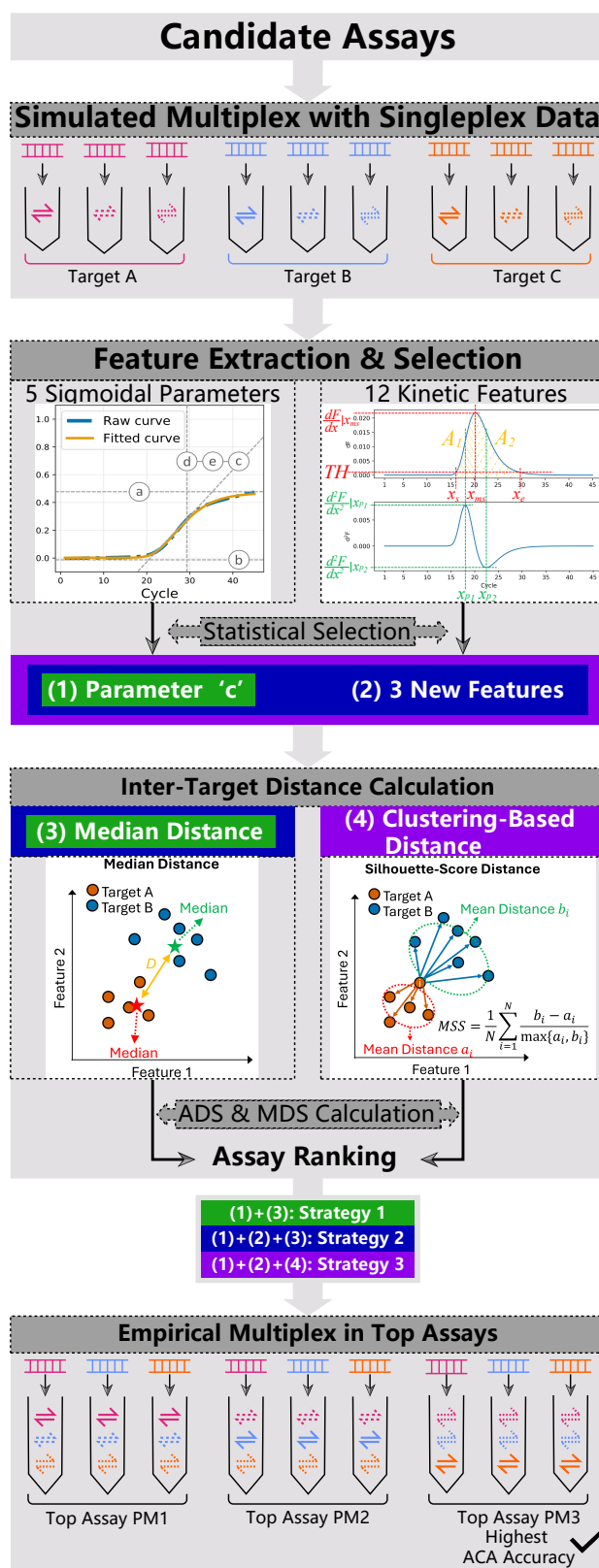


Figure 3.1: Framework of Smart-Plexer and differences between Smart-Plexer 1.0 & 2.0. Smart-Plexer includes four steps: Multiplex Simulation, Feature Extraction & Selection, Inter-Target Distance Calculation, and Empirical Multiplex in Top Assays. Smart-Plexer 1.0 uses only (1) 'c' parameter and calculates (3) median distances among target clusters, ignoring inner-cluster distributions. Smart-Plexer 2.0 leverages (2) three new features along with 'c' (Strategy 2), and (4) Clustering-based Distance which measures whole-cluster proximity (Strategy 3).

In our group's previous research (Smart-Plexer 1.0), a five-parameter asymmetrical sigmoidal model was used to fit the amplification curves, and a single fitting parameter ' c ' (which is the reciprocal of S_c in Equation 2.1), which correlates with the curve slope, was selected to calculate inter-target distances, as shown in Figure 3.1(1) [14], [124]. Although robust and reliable in controlled reactions, this single-feature approach limits performance under complex conditions, such as PCR inhibition, variable primer efficiency, and fluctuating target concentration [13], [141], in which the changes of reaction efficiency will result in distorted AC shapes. In these situations, plateau shifts and altered slopes of curves may distort ' c ' values, potentially compromising the accuracy of inter-target distance measurement. Furthermore, Smart-Plexer 1.0 utilised median distance, as depicted in Figure 3.1(3), which considers only inter-target average distances without addressing inner-target distributions, leading to potential imprecise ADS and MDS. The original reported process combining Step (1) and (3) is referred to as Strategy 1 (S1).

This chapter introduces Smart-Plexer 2.0, an enhanced framework designed to address these limitations and improve assay reliability in challenging scenarios. This new version introduces two key advancements: **1) Enhanced Feature Extraction**: twelve novel kinetic features were developed based on biological insights from PCR fluorescent readouts. Statistical evaluations were then performed to select the optimal features that are most distinguishable across targets, as shown in Figure 3.1(2). These parameters complement the slope-related ' c ' parameter reported in Smart-Plexer 1.0, significantly enhancing the selection process for multiplex assays by introducing additional kinetic information. **2) Advanced Distance Measurement**: a novel method was proposed for distance measurement that considers the inter-target-cluster distances, evaluating relationships between target distributions, as shown in Figure 3.1(4). Integrating these innovations, two new ACA assay selection strategies were established: Strategy 2 (S2), which combines the enhanced feature sets with conventional Median Distance calculation, and Strategy 3 (S3), which leverages enhanced features and Clustering-based Distance calculation. Introducing both S2 and S3 allows for the comparison of different distance measuring methods.

Using two independent retrospective datasets, this chapter demonstrated the robustness of the new features against significant target concentration variations, confirming their stability from singleplex to multiplex settings. Comparing old (S1) and new strategies (S2 and S3) in optimising a 3-plex assay revealed that S2 and S3 outperform S1 statistically, demonstrating superior performance in selecting optimal assays and avoiding unpromising results with a 10-fold reduced Percentile Interval in accuracy distributions for selected top assays. Furthermore, cross-concentration and multi-experiment tests in a newly developed 7-plex assay indicated that

assays selected using S3 showed superior ACA performance, including a 1% improvement in average classification accuracy and an 8.41% narrower accuracy distribution, confirming robustness in complex experimental conditions. This advancement in feature extraction and distance measurement strategies enhances the Smart-Plexer framework, marking a significant step forward in developing reliable and efficient multiplex PCR assays for ACA methodologies, strengthening the capability of high-level multiplexing.

3.3 Methodology

This section presents the methods used to develop and validate the enhanced Smart-Plexer strategies. After presenting data used in this study, this section introduces the design and selection & validation of the new features, followed by the description of the new Clustering-based Distance and Smart-Plexer implementation strategies, then summarise the evaluation carried out for comparison between Smart-Plexer 1.0 and 2.0.

3.3.1 Datasets Used in This Chapter

To ensure robustness, both retrospective and newly generated datasets with cross-platform, cross-experiment, and cross-concentration characteristics were employed. Detailed assay descriptions can be found in Section B.1 and Table B.1.

- (1) The 7-plex Respiratory Tract Infection (RTI) dataset (SP1-7-plex-RTI) [94]: Acquired from our previous Smart-Plexer 1.0 research, it contains reactions of human adenovirus (HAdV), human coronavirus OC43 (HCoV-OC43), human coronavirus HKU1 (HCoV-HKU1), human coronavirus 229E (HCoV-229E), human coronavirus NL63 (HCoV-NL63), Middle East respiratory syndrome-related coronavirus (MERS-CoV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in a single fluorescent channel using qdPCR, across up to 5 concentrations (10^3 , 10^4 , 10^5 , 10^6 , 10^7 copies/ μ L), with 36,960 reactions in total. It was used for developing and selecting optimal features from the AC.
- (2) External concentration-efficiency dataset (Ex-Conc-Eff) [140]: In this external open dataset, qPCR reactions were conducted with the MT-ND1 gene over a wide range of input DNA ($3.14 \times 10^1 - 3.14 \times 10^7$ molecules) with amplification master mix quantities ranging from 60% to 100% of the optimal amount. This dataset, which contains 420 amplification curves, produced different reaction kinetics under a variety of DNA concentrations, mimicking the amplification efficiency reduction that happens

during multiplex reactions in which primer sets and probes of several targets are mixed. We leverage this external dataset to validate the robustness and consistency of the proposed new AC kinetic features across concentrations in extreme reaction conditions.

- (3) The 3-plex RTI dataset (SP1-3-plex-RTI) [94]: The three targets were from our previous Smart-Plexer research, including adenovirus (HAdV), human coronavirus HKU1 (HCoV-HKU1) and the Middle East respiratory syndrome-related coronavirus (MERS-CoV). This retrospective dataset, with 62,370 reactions, was involved in this study because all 27 possible combinations of multiplex assays of the three targets were exhaustively tested empirically in multiplex conditions, making it possible to conduct an overall comparison among different assay selection strategies. This previous 3-plex RTI dataset was used for statistical evaluation of the previous and the new Smart-Plexer strategies over different AC features and distance calculations.
- (4) New 7-plex RTI dataset (SP2-7-plex-RTI): To further prove the effectiveness of the improved Smart-Plexer framework, we applied both the original and proposed new Smart-Plexer strategies on a newly developed multiplex panel of 7 respiratory infectious pathogens: influenza A virus H1N1 2009 or swine flu (A/H1N1/09), influenza A virus H1N1 (A/H1N1), influenza A virus H3N2 (A/H3H2), influenza A virus (hIAV), influenza B virus (hIBV), respiratory syncytial virus (hRSV), and *Streptococcus pneumoniae* (Spn). The candidate primer sets and probes (1-3 per target) were designed for each target, resulting in 128 possible multiplex combinations. Experiments were first conducted in singleplex with qdPCR for each assay to get the simulated multiplex AC required for Smart-Plexer, generating 34,650 ACs in total. After applying the old and new Smart-Plexer strategies, six optimal multiplex combinations were selected, for which single-well multiplex reactions were performed in qdPCR with a range of different target concentrations (10^4 , 10^5 , 10^6 , 10^7 copies/ μ L). A total of 18 tests with separate qdPCR chips were conducted for the empirical multiplex, resulting in 665,280 amplification curves. Details of experiment conditions and assays can be found in Section B.1.

To align with our group's research interest, different categories of RTI pathogens were selected for this chapter. Although these respiratory pathogens present with similar early symptoms, they can lead to varying degrees of disease severity and distinct prognoses. Early

and accurate identification enables targeted clinical treatment, improving patient outcomes and supporting effective epidemic surveillance, particularly during pandemics.

The Adaptive Filtering method proposed in Chapter 2 was applied to all the qdPCR datasets mentioned above.

3.3.2 New Features from Amplification Curve

Table 3.1: Definition and description of proposed new features.

Name	Definition	Description
Threshold Distance	$x_e - x_s$	Mathematical: Distance between the two Threshold-crossing locations in the first derivative. PCR curve: Difference between the cycle number (or time) at the beginning of the exponential phase to the beginning of the plateau phase.
First-half Distance	$x_{ms} - x_s$	Mathematical: Distance between the first Threshold-crossing location and the maximum-slope location in the first derivative. PCR curve: Difference between the cycle number (or time) at the beginning of the exponential phase to the cycle number (or time) with the fastest increase of fluorescence value.
Second-half Distance	$x_e - x_{ms}$	Mathematical: Distance between the second Threshold-crossing location and the maximum-slope location in the first derivative. PCR curve: Difference between the cycle number (or time) with the fastest increase of fluorescence value to the beginning of the plateau phase.
Distance Asymmetry Index	$\frac{x_e - x_{ms}}{x_{ms} - x_s}$	Mathematical: Ratio of Second-half Distance to First-half Distance, indicating how asymmetrical the peak is in the two phases of the derivative of the fitted curve. PCR curve: Indication of curve asymmetry between the beginning and ending phase of the reaction.
Peak-shifting Distance	$x_{p2} - x_{p1}$	Distance between the positive and negative peak locations in the second derivative.
A_1	$\sum_{x=x_s}^{x_{ms}} f'(x)$	Integration of the first derivative from x_s to x_{ms} , indicating the area under the first half of the peak.
A_2	$\sum_{x=x_{ms}}^{x_e} f'(x)$	Integration of the first derivative from x_{ms} to x_e , indicating the area under the second half of the peak.
Area Asymmetry Index	$\frac{A_2}{A_1}$	Mathematical: Ratio of A_2 to A_1 , indicating how asymmetrical the area-under-peak is. PCR curve: Indication of curve asymmetry between the beginning and ending phase of the reaction.
Maximum Slope	$\frac{df}{dx} _{x_{ms}}$	Mathematical: Height of the largest peak in the first derivative. PCR curve: The largest fluorescence value increased between two continuous cycles.

Positive Second-derivative Peak Height	$\frac{d^2f}{dx^2} _{x_{p1}}$	Mathematical: Height of the positive peak on the second derivative. PCR curve: The reaction speed at the beginning phase of PCR
Negative Second-derivative Peak Height	$-\frac{d^2f}{dx^2} _{x_{p2}}$	Mathematical: Absolute height of the negative peak on the second derivative. PCR curve: The reaction speed at the beginning of the plateau phase.
Peak Asymmetry Index	$-\frac{\frac{d^2f}{dx^2} _{x_{p1}}}{\frac{d^2f}{dx^2} _{x_{p2}}}$	Mathematical: Ratio of the positive peak height to the negative peak height in the second derivative. PCR curve: Indication of curve asymmetry between the beginning and ending phase of the reaction.

Table 3.1 lists the 12 new features dedicated to this study, detailing their definitions and mathematical/biological descriptions.

The calculation of these features relies on the five-parameter sigmoidal fitting of the amplification curve. This chapter extends Equation 2.1 as follows:

$$F(t, \mathbf{p}) = \frac{F_m}{(1 + e^{-S_c(t - C_s)})^{As}} + F_b, \quad (3.1)$$

$$\mathbf{p} = [F_m, F_b, S_c, C_s, As]^T, \quad (3.2)$$

where t is the amplification time (or PCR cycle), $\mathbf{p}$ is the parameter vector, $F(t, \mathbf{p})$ is the fluorescence value, F_m indicates the maximum fluorescence, F_b indicates the baseline fluorescence, S_c is related to the maximum slope of the AC, C_s is linked to the cycle threshold (C_t) value, As is the asymmetry index. The parameter naming was updated from those in Smart-Plexer 1.0 to show clear kinetic meanings, and in the new equation naming system, the previous ‘ c ’ parameter is therefore called ‘ S_c ’.

In Smart-Plexer 1.0, the S_c (or ‘ c ’) parameter was initially identified as a robust feature when transferring from singleplex to multiplex reaction conditions, and its value was used for evaluating the inter-target distances in our previous research. This parameter, though effective, represented a dimensionality reduction (e.g., relying on a single feature), potentially overlooking additional information that could improve multiplex assay optimisation. The challenge of extending feature sets lies in balancing the enhancement of multi-target classification with the reliability and repeatability of the features, given the inherent variability of PCR-based biological events.

Therefore, this chapter further explored 12 new features extracted from the amplification curve and its first and second derivatives. To make the original and the derivative curves smooth enough for reliably extracting kinetic features, the five-parameter sigmoidal fitting was first

applied to the raw observation of AC. Non-linear Least-Squares optimisations were applied to acquire the optimal estimations of $\mathbf{p}$, which were later used for calculating the first and second derivatives of AC with the following equations [124]:

$$F'(t) = \frac{\partial F(t, \mathbf{p})}{\partial t} = \frac{F_m S_c A_s e^{-S_c(t-C_s)}}{(1 + e^{-S_c(t-C_s)})^{A_s+1}}, \quad (3.3)$$

$$F''(t) = \frac{\partial^2 F(t, \mathbf{p})}{\partial t^2} = \frac{F_m S_c^2 A_s e^{-S_c(t-C_s)} (A_s e^{-S_c(t-C_s)} - 1)}{(1 + e^{-S_c(t-C_s)})^{A_s+2}}, \quad (3.4)$$

Figure 3.2(a) illustrates a raw amplification curve, its fitted curve, and the corresponding first and second derivative shapes, highlighting fiducial points and extracted features. The raw curve is precisely fitted and smoothed while kinetic information is kept. The maximum peak location and height of $F'(t)$ are picked as x_{ms} and $\frac{dy}{dx} \big|_{x_{ms}}$. By setting a small threshold TH on the first derivative, the TH -crossing points x_s and x_e can be identified, representing the beginning of the exponential phase and the beginning of the plateau phase, respectively. Locations of both the positive and negative peaks of the second derivative were also extracted as x_{p1} and x_{p2} , which are linked with the accelerating and plateauing period of AC.

Provided these fiducial points, 12 new features were designed and extracted as detailed in Table 3.1. A group of features focuses on measuring the increasing speed of fluorescence before saturation, e.g., $x_e - x_s$, $x_{p2} - x_{p1}$, $\frac{df}{dx} \big|_{x_{ms}}$, $\frac{d^2f}{dx^2} \big|_{x_{p1}}$, $\frac{d^2f}{dx^2} \big|_{x_{p2}}$, whereas others aim to evaluate the degree of asymmetry between the exponentially increasing phase (period before x_{ms}) and the plateauing phase (period after x_{ms}), e.g., $\frac{x_e - x_{ms}}{x_{ms} - x_s}$, $\frac{A_2}{A_1}$, $-\frac{\frac{d^2f}{dx^2} \big|_{x_{p1}}}{\frac{d^2f}{dx^2} \big|_{x_{p2}}}$.

Instead of looking only at S_c ('c' parameter in previous publications, which is related to maximum slope), this set of new features describes the AC from multiple perspectives, and takes not only the slope but also the higher-level changing trends and asymmetry into consideration, providing a wider range of kinetic information. These 12 features, together with the fitted five parameters $\mathbf{p}$, pave the way for the following steps of optimising the Smart-Plexer strategy.

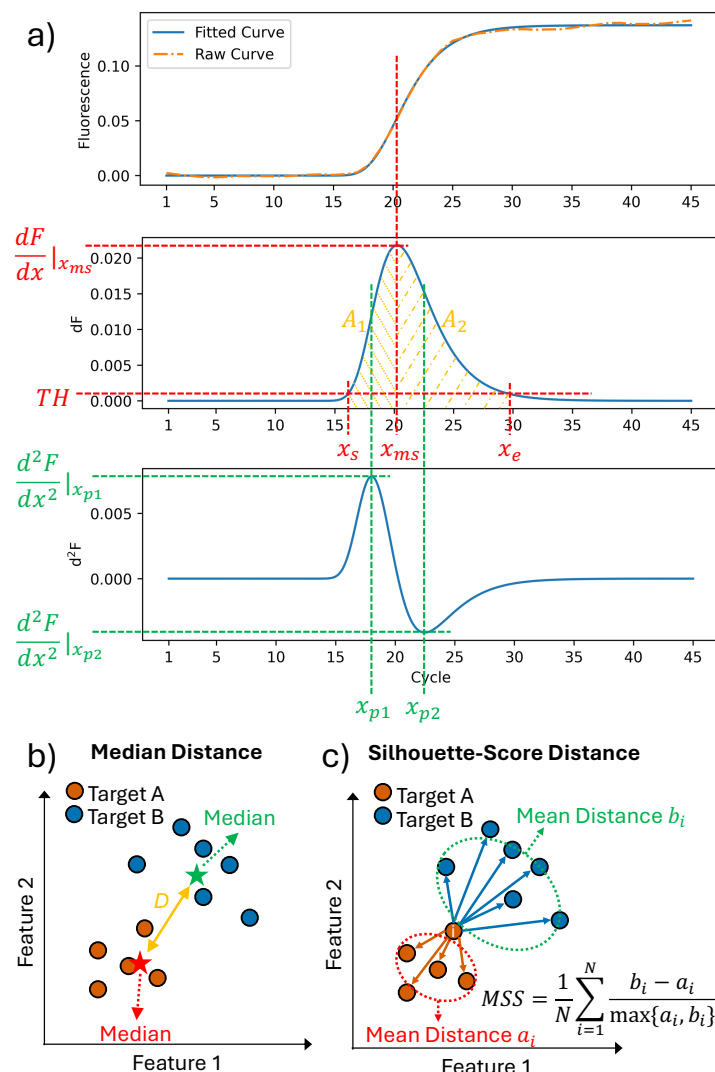


Figure 3.2: Key methodology overview of Smart-Plexer 2.0. (a) Extraction of fiducial points and kinetic features from the fitted amplification curve (top), first derivative (middle), and second derivative (bottom). (b) Median Distance calculation using median points of targets in feature space. (c) Calculation of clustering-based Mean Silhouette Score (MSS), using both intra- and inter-target Euclidean distances to measure cluster separation between target A and B.

3.3.3 Feature Selection and Validation

Within the 12 new features plus the five parameters (17 features in total), we need to select and validate those satisfying the following criteria:

Criterion 1: Features should be significantly distinguishable across targets; in that case, they could be used to calculate ADS and MDS, which are vital for the Smart-Plexer workflow.

Criterion 2: Features should be robust across concentrations under a wide range of reaction efficiencies; in that case, the unknown sample concentrations will not affect the features' distributions.

Criterion 3: Feature distributions should be kept or at least linearly correlated when transferring the reaction condition from singleplex to multiplex. In that case, the ADS and MDS of simulated and empirical multiplexes will remain strongly correlated. Thus, using singleplex-simulated ADS and MDS will not affect the features' capability of predicting optimal assays, which is vital for Smart-Plexer to work.

To select based on **Criterion 1**, the 17 features were extracted on the SP1-7-plex-RTI dataset, and Mean Silhouette Scores (MSS) were calculated for each feature across all seven targets using the following equation [135], [142]:

$$\text{MSS} = \frac{1}{N} \sum_{i=1}^N \frac{b_i - a_i}{\max\{a_i, b_i\}}, \quad (3.5)$$

where N is the total number of amplification curves in the dataset after AMF, and for each data point i , a_i is the mean of the Euclidean Distances between i and all other data points within the same target category as i . b_i is the mean of the Euclidean Distances between i and all the data points within the specific target category which is the closest to i . Here, groupings were performed with nature labels of targets when calculating MSS. The closer the inner-target data points and the farther the inter-target data points are, the higher the MSS value will be. Therefore, a feature with a higher MSS value indicates it is easier to distinguish targets using this feature, and is optimal for calculating ADS and MDS.

Secondly, to validate the cross-concentration robustness of the features, analyses were applied to each of the 17 features extracted from the Ex-Conc-Eff dataset, where the features' distribution consistency across seven concentrations under decreasing reaction efficiencies was evaluated using the Kruskal-Wallis test [143], which is the non-parametric version of ANOVA. Each concentration group is considered independent observations of feature values, and the Kruskal-Wallis test was applied on seven groups of observations to detect significant differences in data distributions across groups.

Lastly, the correlation between the features of singleplex and multiplex reaction conditions was analysed. We leveraged the SP2-7-plex-RTI dataset since it has both observations from singleplex and multiplex reactions. For each primer set (including the probe) and a given feature, we calculated the feature values from the median curves of both the given primer set's singleplex and the corresponding multiplex reactions. Please note that in multiplex reactions, the given primer set is mixed with other targets' primers, resulting in a potentially lower reaction efficiency because of additional thermodynamic interactions [10], [144]. Correlation

analyses, including regression plots and Pearson Correlation Coefficients (R), were applied to these paired singleplex and multiplex features.

3.3.4 Clustering-based Distance Measurement and New Smart-Plexer Strategies

Using the selected feature vector, this chapter introduces a Clustering-based Distance Measurement approach to calculate distances between different targets. Previously, as shown in Figure 3.2(b), distances were measured using only average (median) values, neglecting inner-target distribution variations. This limitation could result in artificially large distance values even if clusters overlap or have significant internal variance, causing imprecise ADS and MDS calculations. To overcome this, we adopted MSS as the distance measurement among clusters of feature vectors as illustrated in Figure 3.2(c), considering both inter- and intra-target distributions, thus providing a comprehensive representation of relationships between target clusters and ensuring more precise ADS and MDS calculations. Data points of feature vectors were clustered based on target labels.

Next, three strategies for the Smart-Plexer workflow were proposed:

- (1) Strategy 1 (S1): The original Smart-Plexer 1.0, where the feature vector has only one element, the 'c' parameter, and median distances were used for deriving ADS and MDS.
- (2) Strategy 2 (S2): The feature vector contains all the selected features, and median distances were used for deriving ADS and MDS. Note that we standardised the mean of each feature to 0 and the standard deviation (STD) to 1 before calculating distances.
- (3) Strategy 3 (S3): The feature vector contains all the selected features, and clustering-based distances were used for deriving ADS and MDS. Note that we standardised the mean of each feature to 0 and the STD to 1 before calculating distances.

3.3.5 Evaluation of New Strategies

The performance of S2 and S3 vs S1 was compared using the SP1-3-plex-RTI dataset, with which we can test in-silico on all the assay combinations. The three strategies were applied to yield optimal primer mixes, respectively, and the ACA performance in classifying three targets was evaluated using the method described in 2.3.8, with 30-fold cross-validation. By analysing the classification accuracies of each panel (which includes 770 AC in the qdPCR platform), new strategies can be compared to the original one.

Furthermore, S1 and S3 were chosen to help develop a new 7-plex assay (SP2-7-plex-RTI). Using the singleplex data, the two strategies generated their own measurement of ADS and MDS, based on which we selected the top 3 combinations for each strategy and tested empirically in multiplex conditions. ACA using leave-one-concentration-out (LOCO) was then conducted on all the chosen primer mixes, and statistical analyses were applied to the accuracies of each panel to determine the advantages of S3 over S1. We demonstrated this cross-concentration multi-experiment test to validate the new strategy's performance under the influence of complex experimental factors.

3.3.6 Algorithm Implementation

Data analysis was conducted on a MacBook Pro (M1 Pro, 32GB RAM, Apple Inc.) with Python 3.9.17. Requirements for key packages of the development environment are provided in Table B.2.

3.4 Results

3.4.1 Selected Features and Their Robustness

The feature distributions across concentrations on the SP1-7-plex-RTI dataset are presented in Figure B.1 and partially depicted in Figure 3.3(a). Most features show large inner-target variances and small inter-target distances, complicating target classification based on them. However, several features, including $\frac{df}{dx}|_{x_{ms}}, \frac{d^2f}{dx^2}|_{x_{p1}},$ and $-\frac{d^2f}{dx^2}|_{x_{p2}},$ exhibited significantly narrower intra-target distributions and clear inter-target distinctions. This intuitive impression was endorsed by their considerably higher MSS, where $\frac{df}{dx}|_{x_{ms}}, \frac{d^2f}{dx^2}|_{x_{p1}}, -\frac{d^2f}{dx^2}|_{x_{p2}}, x_{p2} - x_{p1}$ are showing more than twice the MSS values of all other features, along with S_c whose efficacy was discussed in Smart-Plexer 1.0. $\frac{df}{dx}|_{x_{ms}}, \frac{d^2f}{dx^2}|_{x_{p1}}, -\frac{d^2f}{dx^2}|_{x_{p2}}$ are new features correlating to the maximal reaction speed and the higher-level trend of increasing and decreasing during the beginning and ending phase of the reaction, describing changes in reaction efficiency and asymmetry specific to each target; thus, they function as indicators of target differences. $x_{p2} - x_{p1}$ shows good robustness for most targets, but is performing unsatisfactorily for HAdV; therefore, it is excluded from further consideration.

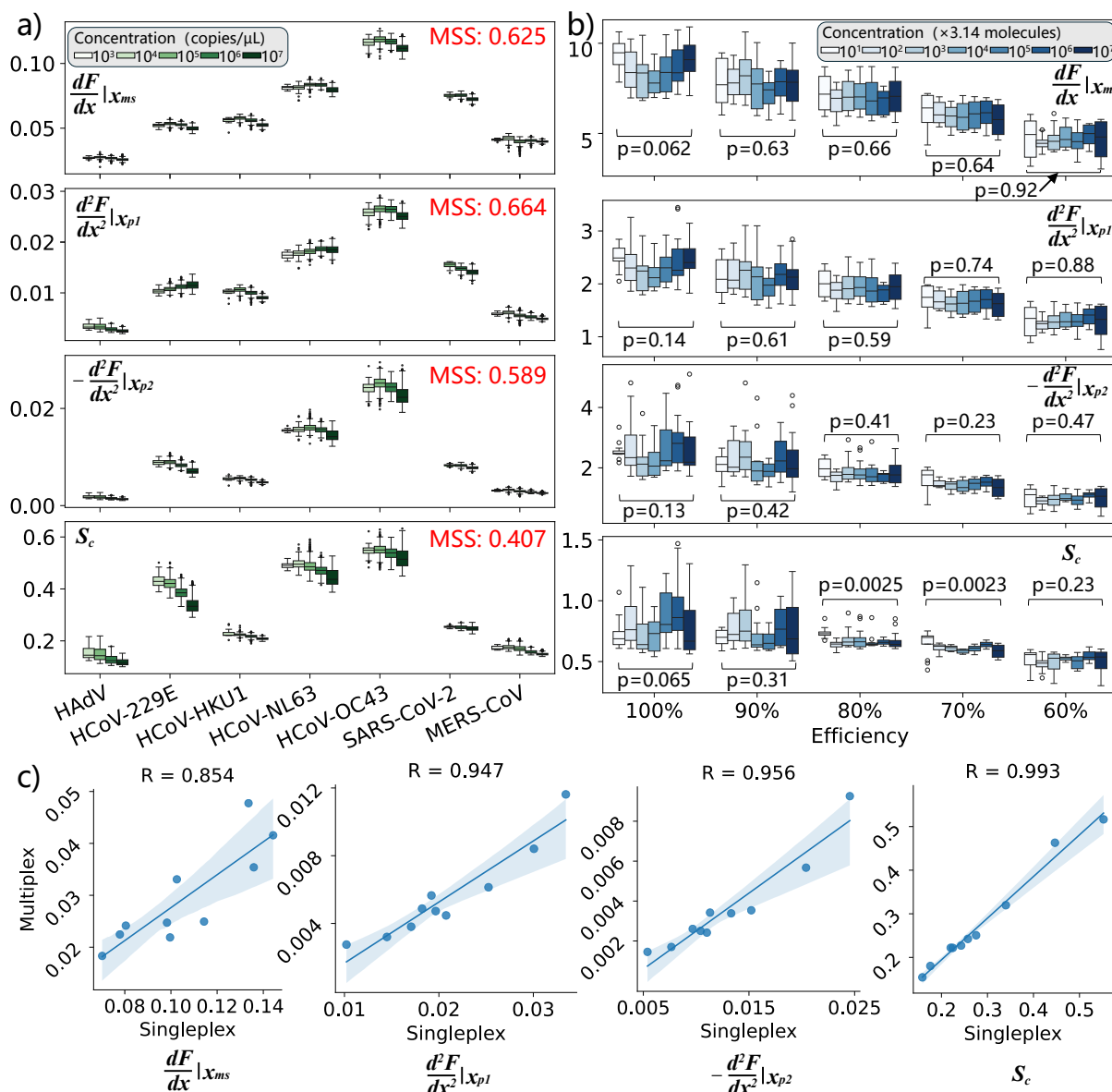


Figure 3.3: Results of feature selection. (a) Box plots of four selected features from the SP1-7-plex-RTI dataset. The X-axis shows target categories, with distributions of target concentrations depicted in colour shades and grouped in subplots. MSS among target clusters are presented. (b) Box plots illustrating feature distributions across reaction efficiencies (X-axis) and concentrations (shaded colours), from the Ex-Conc-Eff dataset, with p -values of Kruskal-Wallis tests among concentrations presented. Standard boxplots were used in (a) & (b) where the box spans from Q1 to Q3, the whisker extends to the 1.5*IQR from Q1 and Q3, and outliers are depicted in dots. (c) Correlation plots and coefficients between Singleplex and Multiplex-extracted features, where translucent bands indicate 95% confidence interval.

The features were tested on the Ex-Conc-Eff dataset, and their distribution consistency among concentrations under different reaction efficiencies can be seen in Figure B.2, a subset of which is depicted in Figure 3.3(b). The three new features demonstrated robustness against concentration variation, as shown by Kruskal-Wallis tests (p -values significantly above the 0.01 threshold), confirming that distributions remain stable across varied concentrations. This

concentration-robustness is valid across all the reaction efficiencies, implying that the features may remain concentration-irrelevant when changing the reaction environment from singleplex to multiplex. Although S_c showed slight sensitivity to substantially low concentrations, indicated by lower robustness in extreme conditions, its performance remained stable in typical conditions (post-hoc Dunn’s tests confirm the most significant differences only between the lowest concentration group and the rest). This emphasises the idea of extending feature dimensions, as a single feature can be unreliable in certain conditions, whereas ensemble strategies avoid the solo dependence on potential extreme values. Considering that S_c has good performance in all other concentrations except the lowest and was previously involved in Smart-Plexer, we kept it in the feature vector for the following tests.

Finally, we analysed the singleplex-multiplex correlations of the selected four features $\frac{df}{dx}|_{x_{ms}}, \frac{d^2f}{dx^2}|_{x_{p1}}, -\frac{d^2f}{dx^2}|_{x_{p2}},$ and S_c , as presented in Figure 3.3(c). All the features correlate with narrow confidence intervals and R values higher than 0.85. S_c shows the highest R, indicating excellent information consistency between single- and multiplex, which was also validated in the previous paper [94]. For most features, the absolute values drop when switching from singleplex to multiplex environments. This phenomenon is consistent with the observations in Figure 3.3(b), giving the assumption that in multiplex conditions, the reaction efficiency for each target is decreasing [10], [144]. However, a drop does not affect the simulated ADS and MDS, as features are standardised before calculating distances. Therefore, simulated ADS and MDS will always show good consistency with their empirical versions.

3.4.2 Evaluation of Three Strategies for Smart-Plexer in 3 Plex

Testing results of S1, S2, and S3 on the SP1-3-plex-RTI dataset are presented in Figure 3.4, in which (a)-(c) show distance maps under S1-S3, plotting locations of primer mixes with simulated ADS and MDS on the x- and y-axis, respectively [94]. Top combinations with both high ADS and MDS have distinguishable AC, clustering towards the map’s top-right corner, while less optimal combinations cluster at the bottom-left. We also plotted the empirical ACA classification accuracy in colour shades on the maps.

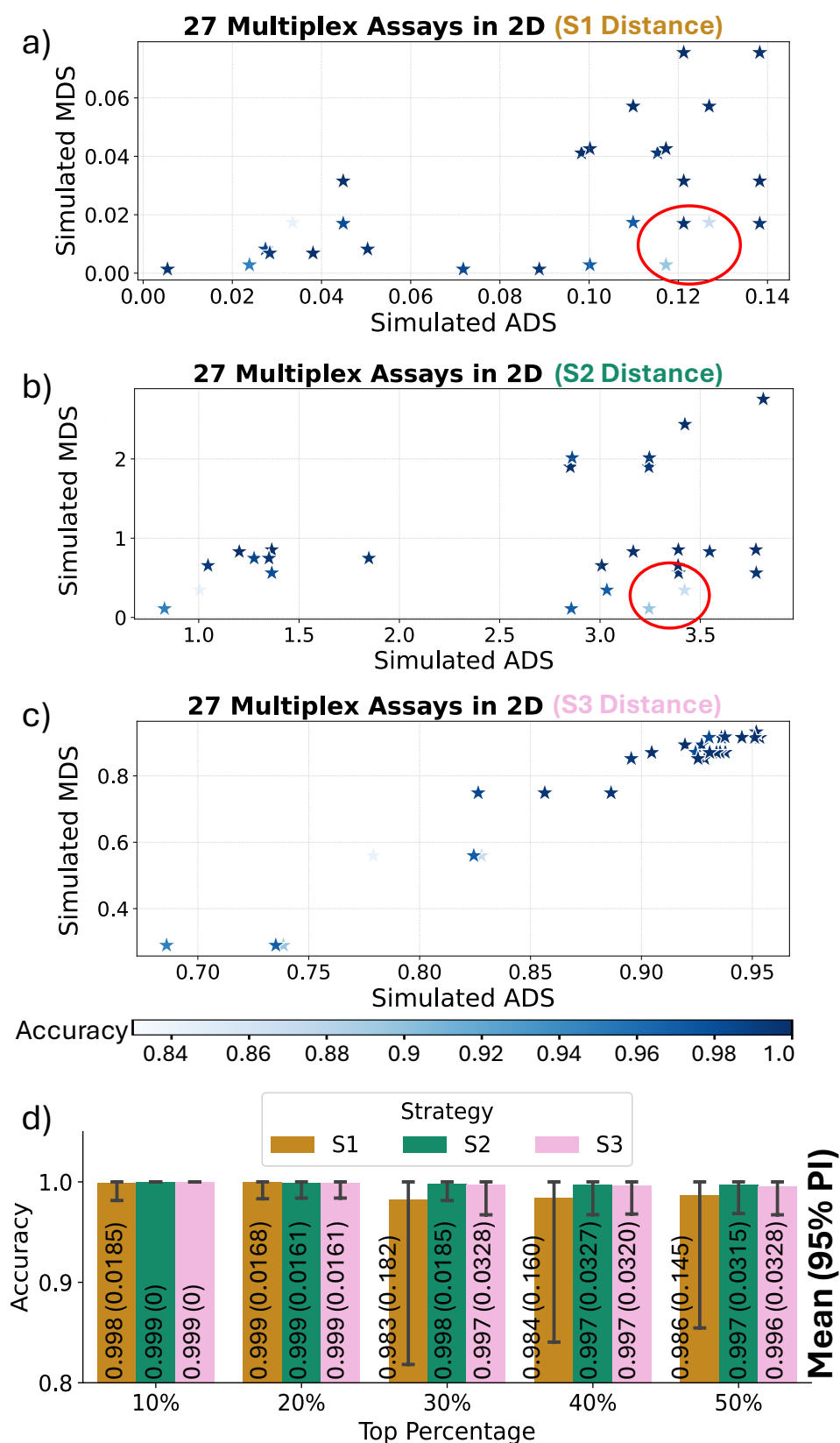


Figure 3.4: Distance maps under (a) S1, (b) S2, and (c) S3. ACA accuracies of multiplexes are marked with shades of colours. Multiplexes with unexpectedly low ACA accuracies are circled in (a) and (b). (d) Bar plots of ACA accuracies when picking the top 10%-50% assays using the three strategies, with the mean of panel accuracies shown by the bar height and 95% PI demonstrated by the error bar.

Most assays provide high performance of ACA, probably due to the relatively simple task of classifying only three targets. However, certain assays predicted by S1 and S2 as high-performing (right side of maps, Figure 3.4(a)-(b)) empirically yielded lower accuracy (<90%), highlighting inconsistencies between ADS-MDS predictions and empirical outcomes. Such inconsistency can result in unexpectedly low accuracy in the selected optimal assays, potentially harming the overall stability of Smart-Plexer. The S3, with the newly proposed MSS distance measurement technique, shows a unique pattern where all the high-accuracy assays are located in the upper-right corner and are keeping adequate distances from the bottom ones, as depicted in Figure 3.4(c). This sparse distance pattern benefits the top-assay selection by limiting the chance of highly ranking unsatisfactory combinations showing up.

Quantitative performances are shown in Figure 3.4(d), in which the mean and 95% Percentile Interval (PI) of panel accuracies when picking the top 10%-50% assays using the three strategies were provided. Although the top 10%-20% selections of S2 and S3 do not show significant improvement in mean accuracy compared to S1, they do have smaller PI. When selecting the top 30%-50%, S2 and S3 consistently outperformed S1, achieving higher mean accuracies and significantly narrower PIs, confirming enhanced reliability of assay selection with a higher chance of picking optimal assays. The ranking-accuracy mapping presented in Figure B.3 also strengthens the assumption that increasing feature dimensions and optimising distance calculation will ensure a higher chance of picking good combinations and avoiding unpromising ones. In Figure B.3, S2 and S3 tend to have low-accuracy assays ranked lower, whereas S1 may accidentally promote low-performance combinations to the top, harming the overall robustness of it.

3.4.3 Developing a New Multiplex Assay

In the previous section, it was concluded that S2 and S3 can provide more reliable performance in assay selection than S1. This chapter further validated these strategies on a new 7-plex development, generating the SP2-7-plex-RTI dataset. The four top primer mixes (PM7.13, PM7.14, PM7.77, PM7.78) of S1 and the three top primer mixes (PM7.14, PM7.16, PM7.80) of S3 were selected, respectively, based on the singleplex data. Although we aimed to select the top three for each strategy, two primer mixes were ranked in third place simultaneously by S1, which is possible according to the ranking system proposed in Smart-Plexer 1.0. It can also be seen that PM7.14 is ranked top by both strategies.

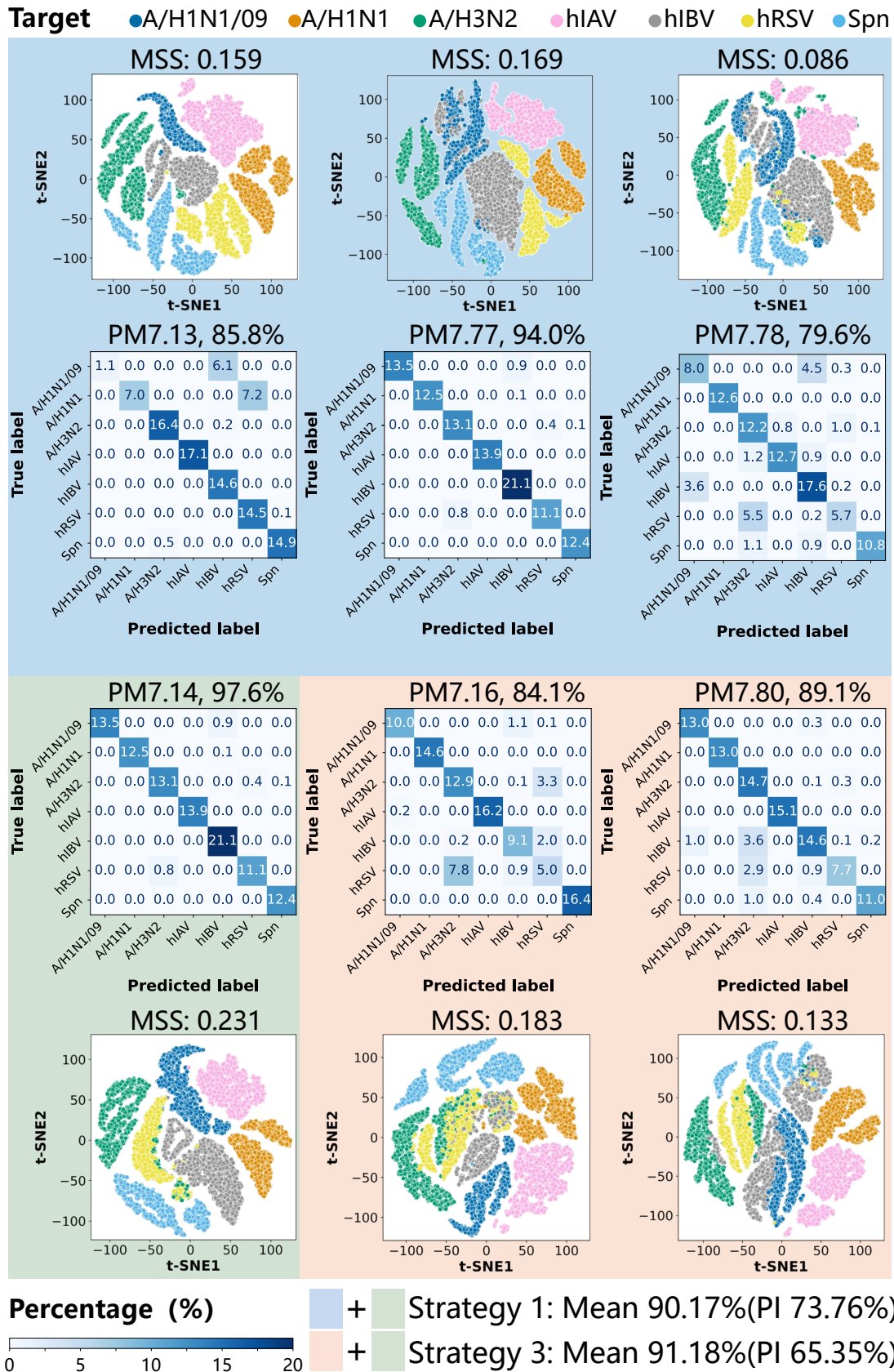


Figure 3.5: ACA performance of assays selected by S1 and S3 on SP2-7-plex-RTI dataset. S1-picked, S3-picked, and commonly picked assays are marked in different colours. For each assay, the confusion matrix with the ACA accuracy and the t-SNE mapping with the Mean Silhouette Scores of target clusters are presented in a stacked way.

Figure 3.5 illustrates the detailed ACA classification performance for selected assays through confusion matrices and corresponding t-distributed Stochastic Neighbor Embedding (t-SNE) mappings [145]. The classification accuracies and MSS of the t-SNE mapped features are given in the plot titles. Clusters were mapped by t-SNE, and the colours were presented with target labels. t-SNE technique visualises the feature distributions using manifold learning, with smaller cluster contours and larger inter-target cluster distances indicating easier separability by non-linear classifiers. Here, MSS evaluates clustering quality, with higher MSS reflecting better-defined clusters, indicating easier ACA classification with the given assay.

As depicted in Figure 3.5, PM7.14 demonstrates the highest target identification accuracy, with most samples falling into the diagonal of the confusion matrix. Also, its t-SNE mapping separates target clusters, reflecting distinct boundaries and large inter-cluster distances. PM7.78 shows the opposite performance, with ACA accuracy less than 80% and MSS close to zero (indicating that clusters are indifferent). In its t-SNE mapping, broken clusters of A/H1N1/09 and hRSV can be spotted. All the other primer mixes have accuracies between 84.1% and 94%, and on average, the S3 picked assays are showing better and more robust performance, with over 1% higher mean ACA accuracy, 8.41% smaller PI, and larger mean MSS (0.161 for S1 vs 0.182 for S3).

3.5 Discussion

3.5.1 Improvement of Smart-Plexer 2.0

This chapter aims to enhance Smart-Plexer 1.0 by incorporating additional kinetic features alongside the ‘c’ parameter and introducing a clustering-based distance calculation, thereby improving its robustness under varying experimental conditions such as concentration fluctuations and PCR inhibitors. Results indicate that while the S1 strategy can still identify top-performing primer mixes, the expanded feature set and clustering-based distance measurement of S3 provide a more stable and reliable prediction of optimal combinations. Incorporating comprehensive kinetic and target-distribution information significantly increases the likelihood of selecting high-performance assays while avoiding less promising combinations. This improvement is evident in the reduced interval between the maximum and minimum classification accuracy (18% accuracy difference for S1 and 13.5% for S3), demonstrating a more consistent performance.

Practically, multiplex assay design faces challenges due to limited empirical testing capacity resulting from cost and time constraints. Smart-Plexer 2.0 addresses these challenges by efficiently and accurately identifying promising primer combinations *in silico*, significantly streamlining assay development. This advancement offers a tangible benefit in diagnostics, where rapid and precise assay design is crucial.

Comparing with existing curve-derived sigmoidal parameters, the selected features show significant advantages: (1) they are calculated on top of the five-parameter model, presenting complex non-linear relations inside AC, also avoiding notches and artefacts which usually appear in high-level features; (2) they incorporate both slope (first-derivative) and second-derivative changing trends, taking asymmetry between exponential and plateauing phases into consideration; (3) they are explainable from the perspective of molecular biology, enabling further analysis and optimisation of chemistry.

3.5.2 Considerations in Clinical Applications

The proposed strategy demonstrates a moderate increase in ACA accuracy, as the assays are already well-designed with high benchmark accuracy, leaving little room for further improvement in the numbers. However, statistical tests show clear significance, making the comparison results reliable. In clinical practice, ACA in qdPCR uses a “panel ensemble” strategy, where predictions from all wells within a panel are combined for final sample classification. As we will show in Chapter 4, this ensemble strategy will improve ACA accuracy by 4-14%. Thus, the best assays in Figure 3.5 have the potential to achieve 100% sample-level accuracy with qdPCR in real clinical applications.

Explainable models like Random Forest can effectively indicate feature importance when training and testing data distributions align. However, since our singleplex PCR data differ significantly in distribution from multiplex data, showing correlation but not numerical equivalence, such models can become biased, causing inconsistent and suboptimal feature selection. To address this domain discrepancy, we instead evaluated features directly using clustering and statistical tests, ensuring robust feature selection without domain-shift bias.

Theoretically, there may be differences between data from single-molecule qdPCR and conventional bulk-reaction qPCR (multiple molecules per reaction). Although most results presented were obtained using qdPCR, we anticipate comparable performance of features with qPCR (retraining of classification models may be required), given that these features consistently demonstrated concentration robustness in Dataset Ex-Conc-Eff, which included external qPCR data.

Simulating multiplex with singleplex reactions can introduce artefacts and reduced efficiency due to primer interactions. To address this, NTC tests were performed to eliminate primer dimers and non-specific products. Previous work showed that although feature values may shift between singleplex and multiplex, carefully selected features preserve inter-target distance information through strong linear correlations [94]. This is also validated in Figure 3.3(c) for all chosen features, ensuring that target distances remain consistent and that selected assays remain effective.

3.5.3 Limitations and Future Directions

First, the accuracy of Smart-Plexer’s predictions heavily depends on the quality of input data from singleplex assays. Inaccuracies may yield suboptimal multiplex designs. Second, although designed for multiplex PCR complexity, Smart-Plexer 2.0 still encounters challenges with highly complex interactions, such as primer competition or non-specific amplification, which are challenging to predict *in silico*. Further improvements, including detailed modelling of primer interactions and *in-silico* simulations incorporating PCR inhibitors, are required to address these issues [13], [146].

Additionally, this chapter primarily focuses on assay development, without extensive investigation into the detection limits for ultra-low concentration targets or the performance with high-GC content templates. For ultra-low target concentrations, sensitivity is fundamentally constrained by amplification efficiency and stochastic effects at low copy numbers, potentially requiring further optimisation of primer/probe design or pre-amplification strategies. For high-GC samples, inefficient denaturation can adversely affect performance; thus, future work will systematically evaluate these challenges and explore protocol modifications to improve assay robustness.

Furthermore, the computational demands of Smart-Plexer 2.0, particularly with the incorporation of new features and advanced distance measurements, can be intensive, requiring substantial processing time and resources. Table B.3 and Table B.4 summarise the running time for each step of the Smart-Plexer and ACA algorithms. On average, Smart-Plexer requires 4.6ms per reaction under Strategies 1 and 2, and 10.8ms under Strategy 3 due to the more computationally intensive clustering distance calculations. For ACA, generating a target prediction for each selected assay takes only 5.5ms. Although it is practical for real-time clinical applications under the provided computing environment, the intensive calculation required can limit its scalability and practicality in resource-limited laboratory settings. To mitigate this, we are working on transforming Smart-Plexer 2.0 into a cloud-based software solution that

optimises memory usage, allowing users to handle all computational tasks through an internet connection, thereby broadening its accessibility and ease of use in diverse laboratory environments.

Lastly, it is natural to consider adapting Smart-Plexer 2.0 to the multiplex assay design of real-time LAMP. However, as discussed in 1.3.5, shapes of LAMP AC across different targets exhibit high similarity due to the rapid reaction kinetics, leaving limited room for optimal assay selection. Therefore, further modulations to the chemistry setups are necessary to first produce distinguishable ACs before any optimal selection strategy can be implemented. This concept and the associated methodology are further detailed in Chapter 6.

3.6 Conclusion

The chapter presented significant advancements in developing and optimising multiplex PCR assays, particularly through enhancing the Smart-Plexer framework. By introducing new features extracted from amplification curves, such as those related to reaction efficiency and curve asymmetry, the study addressed the limitations of Smart-Plexer 1.0, which relied merely on the ‘*c*’ parameter. New features have demonstrated robustness across various concentrations and reaction efficiencies, which are crucial for reliable assay development under multiplex conditions. Clustering-based distance measurement for ADS and MDS calculations was also proposed, aiming for a more inclusive consideration of inter-target distances.

The improved Smart-Plexer 2.0 framework, with its extended feature set and clustering-based distance measurement, outperforms the original version by providing a more stable and reliable prediction of optimal primer combinations. This enhanced approach improves the consistency of assay selection and increases the likelihood of identifying high-performing assays while minimising the risk of selecting suboptimal ones. The successful application of this methodology in developing a new 7-plex assay for respiratory tract infections further validates its practical utility.

In conclusion, Smart-Plexer 2.0 offers a powerful tool for the rapid and accurate design of multiplex assays, addressing the challenges posed by target concentration fluctuations and PCR inhibitors. This advancement is particularly valuable in diagnostic settings, where time and accuracy are essential, and it represents a significant step forward in the field of multiplex PCR assay development.

Chapter 4 Deep Domain Adaptation: Enhancing Cross-Domain ACA

4.1 Chapter Overview

In the last chapter, we successfully developed Smart-Plexer 2.0, which guides the selection of multiplex assays, verifying its efficacy in improving ACA trained and tested on data from synthetic DNA and the same qdPCR platform. However, as mentioned in 1.3.3, target classification purely relying on the amplification curve shapes currently faces challenges in real clinics, such as distribution discrepancies between different data sources of synthetic DNA and clinical samples (i.e., training vs testing), or among samples from various sources (i.e., blood, urine, saliva swabs, biopsies). Optimisation of computational models is required to achieve higher performance of ACA classification in multiplex real-time PCR through the reduction of those discrepancies. This chapter proposed a novel transformer-based conditional domain adversarial network (T-CDAN) to eliminate data distribution differences between the source domain (synthetic DNA data) and the target domain (clinical isolate data). The labelled training data from the source domain and unlabelled testing data from the target domain are fed into the T-CDAN, which learns both domains' information simultaneously. After mapping the inputs into a domain-irrelevant space, T-CDAN removes the feature distribution differences and provides a clearer decision boundary for the classifier, resulting in a more accurate pathogen identification. Evaluation of 198 clinical isolates containing three types of carbapenem-resistant genes (*bla_{NDM}*, *bla_{IMP}*, and *bla_{OXA-48}*) illustrates a curve-level accuracy of 93.1% and a sample-level accuracy of 97.0% using T-CDAN, showing an accuracy improvement of 20.9% and 4.9%, respectively, compared with previous methods in [19] and Chapter 2. This research emphasises the importance of deep domain adaptation to enable high-level multiplexing in a single qPCR reaction, providing a solid approach to extend qPCR instruments' capabilities without hardware modification in real-world clinical applications. This research emphasises the importance of deep domain adaptation to enable high-level multiplexing in a single qPCR reaction, providing a solid approach to extend qPCR instruments' capabilities without hardware modification in real-world clinical applications.

The concept and experimental work presented in this chapter resulted in the following published article:

Y. Mao*, K. Xu*, L. Miglietta, L. Kreitmann, N. Moser, P. Georgiou, A. Holmes, J. Rodriguez-Manzano, ‘Deep Domain Adaptation Enhances Amplification Curve Analysis for Single-Channel Multiplexing in Real-Time PCR’, *IEEE Journal of Biomedical and Health Informatics*, vol. 27, no. 6, pp. 3093–3103, Jun. 2023.

***Joint first authorship, contribution:** conceptualisation, methodology, software, validation, formal analysis, investigation, data curation, writing-original draft, writing-review & editing, visualisation. I served as the primary researcher for this study, handling all data science-related work, including but not limited to algorithm development, coding, model training and tuning, data validation, and result analysis.

Some figures and tables in this chapter and in the corresponding appendix are reproduced or adapted from the abovementioned publication, and are reused with permission from the publisher under the author rights policy: CC BY 4.0 [43].

4.2 Introduction

Previous efforts have demonstrated the promising application of the ACA methodology. However, problems still exist: (1) Unlike some other biomedical signals, the nature of amplification curves is not fully revealed, which brings difficulties to the empirical design of manually extracted features [147]. Therefore, an end-to-end solution with automatic feature extraction capability is required. (2) We noticed a significant difference in the distributions of amplification curve data between synthetic DNA and real clinical samples, that are caused by the inherent complexity of real-world samples, in which not only target pathogen genes but also undesired background genetic materials such as human DNA and other bacteria’s genes exist [102]. While synthetic DNA can be acquired from manufacturers to generate data for ML model training, annotated clinical samples can be difficult to obtain, especially during the emergence of new pathogens, where safety and ethical consent need to be considered. It is ideal that we could train ACA deep classifiers with synthetic DNA data rapidly without the need for having access to clinical samples, which requires additional restrictions such as appropriate safety level facilities, and apply the model directly to unannotated clinical samples in clinical facilities. However, when training an ML model on synthetic DNA data and testing on clinical data, there is a significant performance drop compared to cross-validation results on either synthetic or clinical data. From the ML perspective, the discrepancy between two data distributions can be regarded as domain differences, where synthetic DNA and clinical specimens are from source

and target domains, respectively. An example of these domain discrepancies between different data sources is depicted in Figure 4.1, where amplification curves from clinical isolates are showing smaller FFI and shifted slopes, indicating changes in reaction efficiency and data distributions. In clinical settings, the true identity (true label) of the target domain is not available. Therefore, the detection of multiple targets requires a robust and unsupervised domain adaptation algorithm.

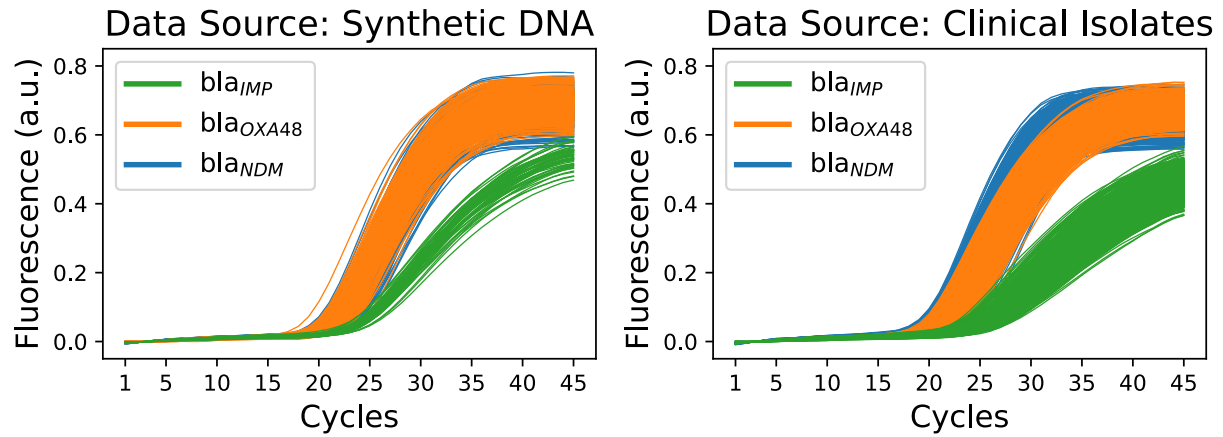


Figure 4.1: An example of domain discrepancies between different data sources. Amplification curves from clinical isolates are showing smaller FFI and shifted slopes, indicating changes in reaction efficiency and data distributions.

Over the past decades, deep learning-based approaches have achieved state-of-the-art performance in various classification tasks. One major advantage of utilising deep learning to categorise time series is that it eliminates the need to extract features manually and instead automatically derives high-dimensional feature representations from data while training [148]. Among the existing deep learning structures, transformers have demonstrated excellent modelling abilities for long-range dependencies and interactions in sequential data, making them ideal for time series analysis [149]. Conditional Domain Adversarial Network (CDAN) [150] is an unsupervised domain adaptation framework that combines the concepts of conditional Generative Adversarial Network (cGAN) [151] and Domain Adaptation (DA). It has emerged as a powerful tool for reducing marginal distribution discrepancies between domains in the vision field [152]. CDAN outperforms other adversarial learning-based DA algorithms (e.g., Domain-adversarial neural networks or DANN [153] and Adversarial Discriminative Domain Adaptation or ADDA [154]) on the alignment of multimodal distributions, allowing it to work better on multi-classification problems.

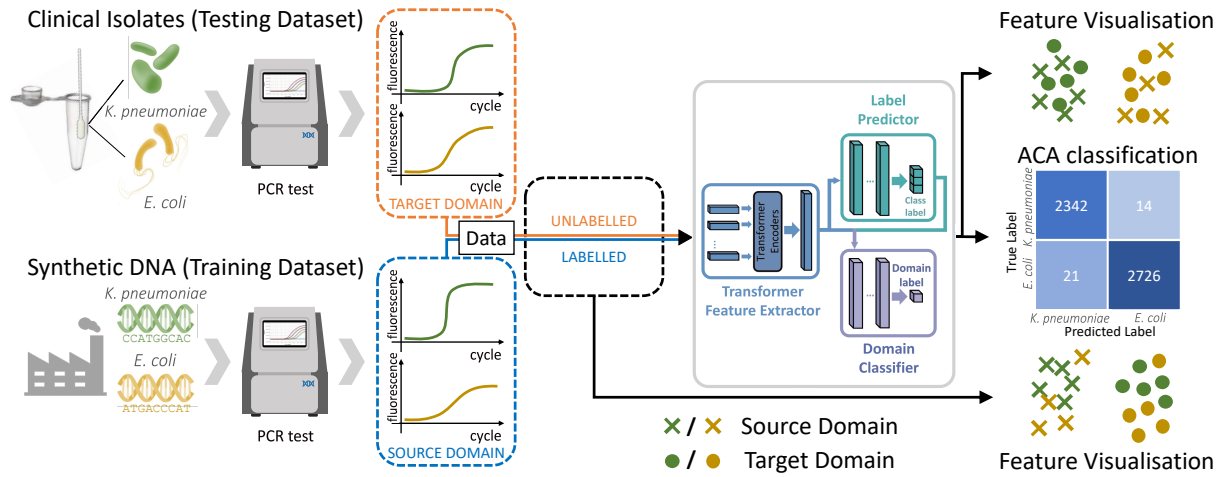


Figure 4.2: Concept workflow of the proposed T-CDAN based strategy. To identify different targets within the collected sample, synthetic DNAs of these targets are ordered from the manufacturer and used to generate the training dataset of the deep learning model. The domain shifts between the training (source domain) and testing (target domain) data cause the distant feature distributions in the two domains, resulting in a dropped performance when applying the source-domain trained model on target-domain data. To eliminate this domain discrepancy, labelled training data and unlabelled testing data are fed into the T-CDAN network, which learns the target and domain information simultaneously. After mapping the inputs into a domain-irrelevant space, T-CDAN can remove the feature distribution differences and provide a clearer decision boundary for the classifier, enabling a better classification result of targets.

This chapter's contributions are two-fold: (1) It introduces a state-of-the-art deep transformer-based network that delivers automatic feature extraction through the attention mechanism to classify amplification curves belonging to different pathogens or targets, providing an end-to-end solution for ACA. (2) A novel transformer-based conditional domain adversarial network (T-CDAN) is proposed to eliminate data distribution discrepancies between the source domain (synthetic DNA data) and the target domain (clinical data).

The overall concept workflow of the proposed strategy is depicted in Figure 4.2. To identify different targets within the collected samples, synthetic DNAs of these targets are ordered from the manufacturer and used to generate the training dataset of the deep learning model. The domain shifts between the training (source domain) and testing (target domain) data cause the distant feature distributions among them, resulting in a dropped performance when applying the source-domain trained model on target-domain data. To eliminate this domain discrepancy, labelled training data and unlabelled testing data are fed into the T-CDAN network, which learns the target and the domain information simultaneously. After mapping the inputs into a domain-irrelevant space, T-CDAN can remove the feature distribution differences and provide a clearer decision boundary for the classifier, resulting in a better target identification.

To verify the effectiveness of the methods, T-CDAN was evaluated on 198 clinical isolates containing three types of carbapenemase genes (*bla_{NDM}*, *bla_{IMP}*, and *bla_{OXA-48}*), achieving the curve-level accuracy of 93.1% and the sample-level accuracy of 97.0%. Compared to previously published methods, T-CDAN shows an accuracy improvement of 20.9% and 4.9% at the curve and sample levels, respectively, with clearer target cluster boundaries and fewer inter-domain distribution differences. This is the first work using a deep feature generator to extract high-level amplification curve features for target identifications in multiplex PCR, and it emphasises the importance of deep domain adaptation in tackling the real-world clinical problem of molecular diagnostics.

4.3 Methodology

4.3.1 Data Information

The datasets described in Chapter 2 were also used in this chapter. The dataset includes amplification events from synthetic DNA (gBlocksTM gene fragments, IDT) of three different carbapenemase genes: *bla_{NDM}* (N=18,480), *bla_{IMP}* (N=17,710), and *bla_{OXA-48}* (N=17,710). Synthetic target sequences were used as the training/source domain, and 198 clinical isolates containing these three genes were used as the testing/target domain. A total of 152,460 amplification events (each containing 45 data points as the number of PCR cycles performed) were pre-processed accordingly with the pipeline described in 2.3.

4.3.2 Transformer-based Amplification Curve Analysis

Amplification curves are typical 1D time-series data; nevertheless, extracting hand-crafted features from these curves is largely underexplored. In preliminary experiments, four methods, including KNN, Random Forest, bidirectional Long Short-Term Memory Network (Bi-LSTM), and 1D-transformer, were introduced to generate the benchmark results on the datasets, which is shown in Table C.1. From the table, it can be observed that 1D-transformer provides the highest result before introducing any transfer learning approach. Therefore, a transformed-based method was presented as the backbone to process amplification curves, leveraging its strength in processing bio-signal time series and capability in automatic feature extraction.

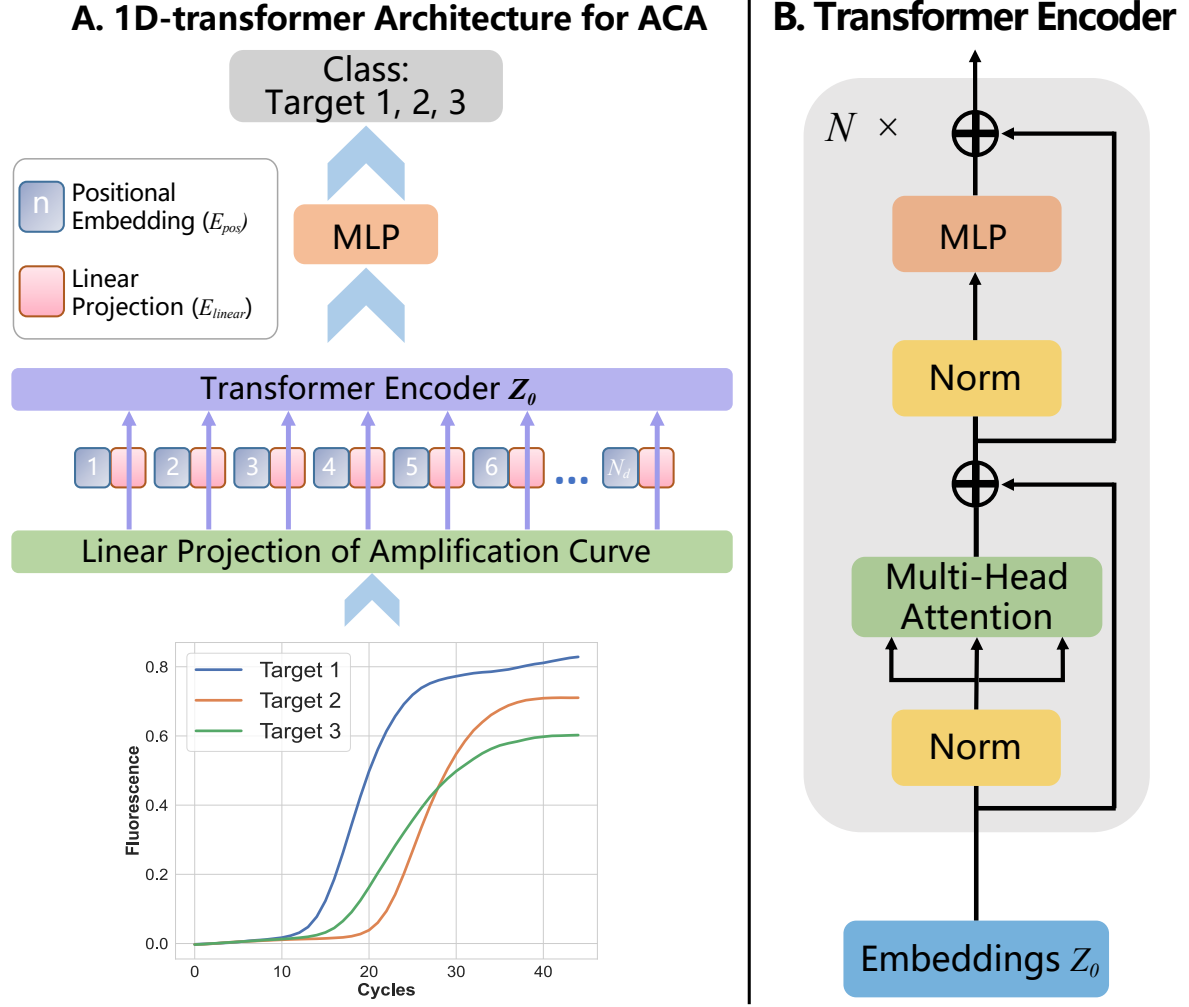


Figure 4.3: Structure of the backbone 1D-transformer. Part A: Illustration of the proposed 1D-transformer network for ACA. The input amplification curve is embedded using the addition of linear projection and positional embedding. The resulting embedding sequence Z is then fed into N conventional transformer encoders, where its detailed structure is depicted in Part B. An MLP network finally utilises the features resulting from the encoders to perform target identification.

An overview of the proposed transformer model for classifying amplification curves is shown in Figure 4.3. We first applied standardisation to set the mean of the data to 0 and the standard deviation to 1. Let $\mathbf{X} \in \mathbb{R}^{C \times 1}$ be a standardised amplification curve, where C is the length of the input curve (i.e., number of cycles). $\mathbf{X}$ is subsequently projected to a N_d -dimensional latent vector using a trainable linear projection layer. Furthermore, because a transformer encoder lacks recurrence or convolution to leverage sequential information, a typical learnable position embedding E_{pos} was element-wise added to the linear projection results E_{linear} , in order to retain essential sequential information within the curve. The resulting

embedding sequence $Z \in \mathbb{R}^{C \times N_d}$ was then fed into N standard transformer encoder layers [155] to extract features (i.e., kinetic information) encoded in the entire amplification curve.

As illustrated in Figure 4.3 Part B, the structure of the transformer encoder block is composed of two sub-layers: (1) multi-head self-attention (MSA) and (2) position-wise Multilayer Perceptron (MLP) sub-layer. To speed up the model convergence and avoid the gradient vanishing problem, a residual connection module followed by a Layer Normalisation (LN) module is inserted around each sub-layer. The representation matrix produced by the i^{th} encoder block is of the consistent size as the input embedding matrix Z_0 .

Instead of following the original Transformer architecture [155], we replaced the decoder part of the transformer with a feed-forward neural network to allow the model to categorise k clinically relevant targets. Mathematically, the entire workflow of the transformer model can be described as below:

$$Z_0 = E_{linear} \oplus E_{pos} \quad (4.1)$$

$$Z'_i = \text{MSA}(\text{LN}(Z_{i-1})) + Z_{i-1}, \forall i = 1, 2 \dots N \quad (4.2)$$

$$Z_i = \text{MLP}(\text{LN}(Z'_i)) + Z'_i, \forall i = 1, 2, \dots N \quad (4.3)$$

$$\mathbf{y} = \text{Softmax}(\text{MLP}(\text{Flatten}(Z_N))) \quad (4.4)$$

where $\oplus$ denotes element-wise addition, Z'_i is the output of the i^{th} MSA block, and Z_i represents the i^{th} point-wise MLP output. Z_N stands for the output of the last transformer encoder layer, and $\mathbf{y} \in \mathbb{R}^k$ indicates the categorical distribution over class labels.

The presented transformer model in this chapter is composed of $N = 4$ transformer encoder blocks, where each of them contains a 4-head self-attention module and a position-wise network with 128 hidden neurons. The expected number of embedding vectors generated using linear projection and a position encoding layer is $N_d = 16$. The presented MLP network consists of three hidden layers, where a batch normalisation and a dropout layer are added in between.

4.3.3 Conditional Adversarial Domain Adaptation for ACA

Let synthetic DNA data be a source domain $\mathcal{S} = \{(x_1^s, y_1^s), \dots, (x_{n_s}^s, y_{n_s}^s)\}$ of n_s labelled amplification curves, where (x_i^s, y_i^s) denotes the i^{th} source domain example, and the clinical isolates dataset be a target domain $\mathcal{T} = \{x_1^t, \dots, x_{n_t}^t\}$ of n_t unlabelled curves, where x_i^t is the i^{th} target domain example. The problem of bridging the domain shift between synthetic DNA and clinical isolates datasets can be intuitively modelled as an unsupervised domain adaptation

problem. In this chapter, T-CDAN was presented to incorporate the aforementioned transformer-based network with the Conditional Domain Adversarial Networks (CDAN) strategy, formally mitigating the discrepancy in data distributions across domains.

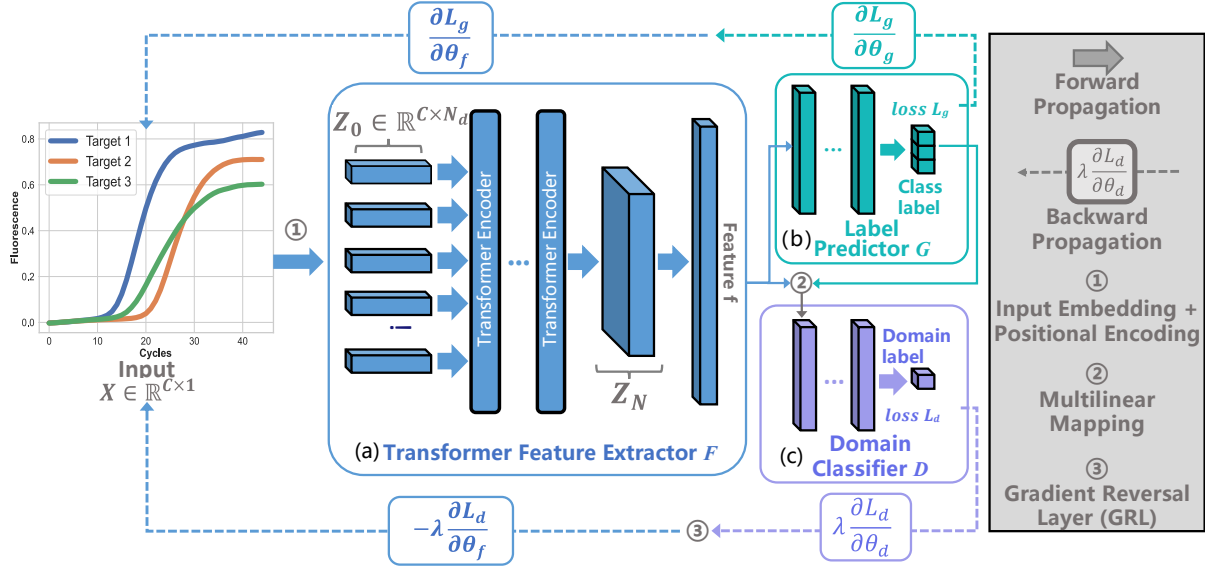


Figure 4.4: Three main components of the proposed T-CDAN framework. (a) Deep feature generator F , (b) label predictor G , and (c) domain classifier D . A sequence of transformer encoder blocks compose F to generate a feature representation $\mathbf{f}$ from a standardised amplification curve $\mathbf{X}$. Label predictor is a standard feed-forward neural network to classify source domain data. D is a two-layer perceptron to determine whether $\mathbf{X}$ is from the source or target domain. Multilinear mapping is applied to condition D on label predictions obtained from G , capturing multi-modal information behind the feature distribution. During the training procedure, the label prediction loss L_g and domain classifier loss L_d obtained from G and D are iteratively minimised, enabling F to learn domain-invariant features finally. The Gradient reversal layer inserted between F and D is utilised to discover shared representations between source and target domains, neatly combining domain-specific and domain-invariant shared features [156].

An overview of the T-CDAN is presented in Figure 4.4, including a transformer-based feature extractor F with parameters θ_f , a label predictor G with parameters θ_g , and a conditional domain classifier D with parameters θ_d . In the forward pass, F extracts domain-specific feature representation denoted as $\mathbf{f} = F(\mathbf{X})$ from input curve $\mathbf{X}$. G takes captured features $\mathbf{f}$ as input to output label predictions $\mathbf{g} = G(\mathbf{f})$. D is a binary domain classifier conditioned by the cross-covariance of $\mathbf{f}$ and $\mathbf{g}$, resulting in a domain label which indicates whether $\mathbf{f}$ is from the source domain or the target domain. Throughout the training procedure, F grows to learn domain-invariant features that can successfully confuse the domain classifier.

D , on the other hand, seeks a rule to distinguish whether the features extracted by F are from the source or target domains.

4.3.3.1 Feature Extractor and Label Predictor

The purpose of the feature extractor F is to learn high-level feature representations from amplification curves, which are implemented in a manner that is consistent with the transformer model described in 4.3.2 without the last MLP network. The output of the last transformer encoder blocks will be reshaped into a vector and utilised directly as the generated feature representation $\mathbf{f}$. The MLP network attached to the transformer encoder block (see Figure 4.3) serves as the label predictor G in the proposed T-CDAN framework. As illustrated in Figure 4.4, F and G comprise a conventional feed-forward architecture. Providing the source and target domain data $\mathcal{S}$ and $\mathcal{T}$, the loss function of the label predictor G is a negative log-likelihood, expressed as:

$$L_g(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g) = -\mathbb{E}_{(x_i^s, c) \sim \mathcal{S}} \sum_{c=1}^{n_c} \tau(y_i^s, c) \log G(c; \mathbf{f}_i^s) \quad (4.5)$$

where $\tau(y_i^s, c) = 1$ if $y_i^s = c$ and $\tau(y_i^s, c) = 0$ otherwise. $\mathbf{f}_i^s$ represents the i^{th} source domain features, $G(c; \mathbf{f}_i^s)$ is the probability of predicting $\mathbf{f}_i^s$ to the c^{th} class, and n_c is the number of classes.

4.3.3.2 Conditional Domain Classifier

The aim of the conditional domain classifier is to distinguish the high-level features in amplification curves across the source and target domains. When we deal with multi-classification problems, the feature distribution is multimodal. In this case, the failure of the classical domain classifier in identifying domain types does not necessarily demonstrate that features which belong to the same class but in different domains have identical distributions, resulting in non-ideal classification performance. Therefore, an ideal domain classifier can only be deceived when it differentiates completely transferable and discriminative features. Inspired by conditional GANs [151], the domain classifier D can capture multimodal structures behind the feature distribution, by conditioning D on the class information included in the label prediction $\mathbf{g}$ when adapting the feature representation $\mathbf{f}$. In particular, the conditioning strategy adopted in D is a multilinear mapping, where we compute the outer product of $\mathbf{f}$ and $\mathbf{g}$, to capture multiplicative interactions between the feature representation and the label prediction:

$$M(\mathbf{h}) = M(\mathbf{f}, \mathbf{g}) = \mathbf{f} \otimes \mathbf{g} \quad (4.6)$$

where $\mathbf{h}$ represents the joint variable of $\mathbf{f}$ and $\mathbf{g}$, and $M(\cdot)$ denotes the multilinear mapping function. Based on binary cross-entropy, the loss function of D is modified and formulated as follows:

$$L_d(\boldsymbol{\theta}_f, \boldsymbol{\theta}_d) = -\mathbb{E}_{x_i^s \sim \mathcal{S}} \log [D(M(\mathbf{h}_i^s))] - \mathbb{E}_{x_j^t \sim \mathcal{T}} \log [1 - D(M(\mathbf{h}_j^t))] \quad (4.7)$$

where $\mathbf{h}_i^s$ and $\mathbf{h}_j^t$ denote the joint variable of $\mathbf{f}_i^s$ and $\mathbf{g}_i^s$, $\mathbf{f}_j^t$ and $\mathbf{g}_j^t$, at the i^{th} source or target domain label prediction, respectively. The proposed domain classifier D , like the label predictor, is a feed-forward neural network with two fully connected layers, each of which comprises 256 neurons. Sigmoid activation is adopted in the output layer, since identifying domain type is a binary classification problem.

4.3.3.3 Optimisation of T-CDAN

During the training stage, the optimal parameters of the feature extractor $\hat{\boldsymbol{\theta}}_f$ and the label predictor $\hat{\boldsymbol{\theta}}_d$ are achieved by maximising $L_d(\boldsymbol{\theta}_f, \boldsymbol{\theta}_d)$, while minimising $L_g(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g)$ leads to the optimal parameters of the domain classifier $\hat{\boldsymbol{\theta}}_g$. It yields adversarial learning between the feature extractor/label predictor and the classifier. Based on the above analysis, the optimisation of T-CDAN can be formulated as a minimax problem of minimising the below overall loss function

$$L(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g, \boldsymbol{\theta}_d) = L_g(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g) - \lambda L_d(\boldsymbol{\theta}_f, \boldsymbol{\theta}_d), \quad (4.8)$$

where $0 \leq \lambda \leq 1$ is a hyperparameter for balancing the two objectives. Motivated by previous work [153], [157], a gradient reversal layer (GRL) is added before the conditional domain classifier, as shown in Figure 4.4. GRL takes no actions in the forward pass, whereas it reverses the gradient when performing a backpropagation operation, which explains why Equation 4.8 uses a minus sign to combine two losses into a single overall loss function. The optimal parameters $(\hat{\boldsymbol{\theta}}_f, \hat{\boldsymbol{\theta}}_g, \hat{\boldsymbol{\theta}}_d)$ can be finally learned by iteratively minimising $L(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g, \boldsymbol{\theta}_d)$. More specifically, $\hat{\boldsymbol{\theta}}_f$ and $\hat{\boldsymbol{\theta}}_g$ can be obtained by addressing the following problem:

$$(\hat{\boldsymbol{\theta}}_f, \hat{\boldsymbol{\theta}}_g) = \underset{\boldsymbol{\theta}_f, \boldsymbol{\theta}_g}{\operatorname{argmin}} L(\boldsymbol{\theta}_f, \boldsymbol{\theta}_g, \hat{\boldsymbol{\theta}}_d). \quad (4.9)$$

Once the optimal parameter $\hat{\boldsymbol{\theta}}_f$ and $\hat{\boldsymbol{\theta}}_g$ are obtained, solve the below problem to search for the optimal parameter $\hat{\boldsymbol{\theta}}_d$ for the domain classifier:

$$(\hat{\theta}_d) = \underset{\theta_d}{\operatorname{argmin}} L(\hat{\theta}_f, \hat{\theta}_g, \theta_d). \quad (4.10)$$

Adaptive Moment Estimation (Adam) optimiser [158] with the $L2$ regularisation of 0.0001 was used to train T-CDAN. The batch size of each domain was 128. As for the learning rate μ , an annealing strategy according to [153] was adopted, defined as $\mu = 0.001(1 + 10p)^{-0.75}$, where $0 \leq p \leq 1$ denotes the training progress, mapped from the first to the last iteration. Note that the learning rate of 15μ was applied to train the domain classifier D , since D has a more lightweight design than the feature extractor G , making it harder to discriminate features compared to G . The hyperparameter λ for balancing the two objectives gradually increases from 0 to 1 using the following formula:

$$\lambda = \frac{1 - e^{-10p}}{1 + e^{-10p}}. \quad (4.11)$$

This method was trained using 10^4 iterations for at least 10 times to reduce the impact of the randomness, and its complete training procedure of T-CDAN for amplification curve analysis is summarised in Algorithm 4.1. Note that the feature distributions over the source and target domains will be unidentifiable for the domain classifier at the end of the training phase, resulting in domain-invariant feature representation.

Algorithm 4.1: T-CDAN Model for ACA - Training Procedure.

Step	Input:	Comment
	$\mathcal{S} = \{(x_i^s, y_i^s)\}_{i=1}^{n_s}$	Labelled amplification curves from synthetic DNA (source domain)
	$\mathcal{T} = \{x_j^t\}_{j=1}^{n_t}$	Unlabelled amplification curves from clinical isolates (target domain)
	$D_s = \{0\}, D_t = \{1\}$	Source and target domain label sets
	λ, μ	Loss weighting and learning rate
	Output:	
	$\hat{\theta}_f, \hat{\theta}_g, \hat{\theta}_d$	Trained parameters
1	Initialise model parameters and learning rate μ	
2	While $< \text{maximum iterations}$:	
3	Compute $L_g(\theta_f, \theta_g)$	Label prediction loss
4	Compute $L_d(\theta_f, \theta_d)$	Domain classification loss

5	$\theta_f \leftarrow \theta_f - \mu(\frac{\partial L_g}{\partial \theta_f} - \lambda \frac{\partial L_g}{\partial \theta_f})$	Update feature generator parameters
6	$\theta_g \leftarrow \theta_g - \mu \frac{\partial L_g}{\partial \theta_g}$	Update label predictor parameters
7	$\theta_d \leftarrow \theta_d - 15\mu\lambda \frac{\partial L_d}{\partial \theta_d}$	Update conditional domain classifier parameters
8	End While	

4.4 Results

4.4.1 Experimental Setup

This chapter focuses on evaluating the performance of the proposed T-CDAN on distinguishing three carbapenemase genes. All codes were implemented in PyTorch.

The proposed one-dimensional (1D) transformer and T-CDAN were first compared with state-of-the-art ML methods for amplification curve analysis: KNN [19] and RF. It is worth noting that KNN used curve-cycle dimensional data as input, whereas RF used hand-crafted features (i.e., sigmoid curve-fitting parameters) as input to the model. 1D-transformer and T-CDAN were trained using the standardised whole curve as inputs.

Over 14,000 double-stranded synthetic DNA reactions were used as the source domain curves, and over 116,000 clinical isolate reactions were used as the target domain curves. T-CDAN followed the standard protocol for unsupervised domain adaptation [153] by using labelled source domain data and unlabelled target domain data for training. All other comparison algorithms solely exploited the source domain data during the training procedure. Feature extractor and label predictor in T-CDAN, together, compose the architecture of the 1D-transformer to fairly evaluate the effectiveness of the domain adaptation.

The two-dimensional t-SNE [145] plot was utilised in this research to visualise the difference between feature representations. Besides, the Proxy $\mathcal{A}$ -distance [157] is used as a metric to assess the discrepancy between the representations of source and target features. It is computed as $dist_{\mathcal{A}} = 2(1 - 2\epsilon)$, where ϵ is the test error of a KNN classifier when distinguishing the source domain from the target domain. Furthermore, because each sample in a panel is divided into 770 microwells and utilised to generate 770 curves in our qdPCR chip, we assessed the models' classification performance at both the curve and sample levels (panel-level). The latter is computed by applying hard votes on predicted positive curve results inside the panel. The model's sample-level classification performance is better than its curve-level performance in

most cases because it ensembles hundreds of independent predictions. This ensemble strategy ensures that the sample will be distinguished correctly as long as the majority of the curves in a panel are correctly classified.

4.4.2 Feature Visualisation

One practical way to compare classification algorithms is to visualise the feature clusters of different targets. The first two columns of Figure 4.5 show the t-SNE plots colour-labelled by domains and targets, respectively. When looking at the overall clustering of plots, only the features generated from T-CDAN can form three recognisable clusters with no apparent domain shift.

The domain-labelled plots in Figure 4.5(a)-(d) (column 1) illustrate the shift between source and target feature representations. The less overlapping area of the two domains' feature distributions shown in the plot, the more significant the domain gap is. Circled areas in Figure 4.5(a)-(c) show that, without the domain adaptation method, there is at least one visible domain discrepancy between the source and target domains. However, as compared to the RF and KNN techniques, the 1D-transformer can yield more domain-overlapping features. This shows that the features captured from the transformer attention mechanism are more domain-invariant than the hand-crafted features. Figure 4.5(d) demonstrates that both source and target domain features generated from T-CDAN have no apparent domain shift and can be projected at relatively consistent locations in 2D t-SNE space. This can be explained by the fact that non-domain adaptation-based classifiers learnt from the source domain data will be biased by the source distribution and adjusted only according to these data. The T-CDAN, on the other hand, focuses on the feature extractor to consider the unlabelled distribution of both source and target domains, resulting in a more generalised mapping from raw curves to the feature space, with a unified distribution of features and no significant density differences, as shown in the first column of Figure 4.5(d).

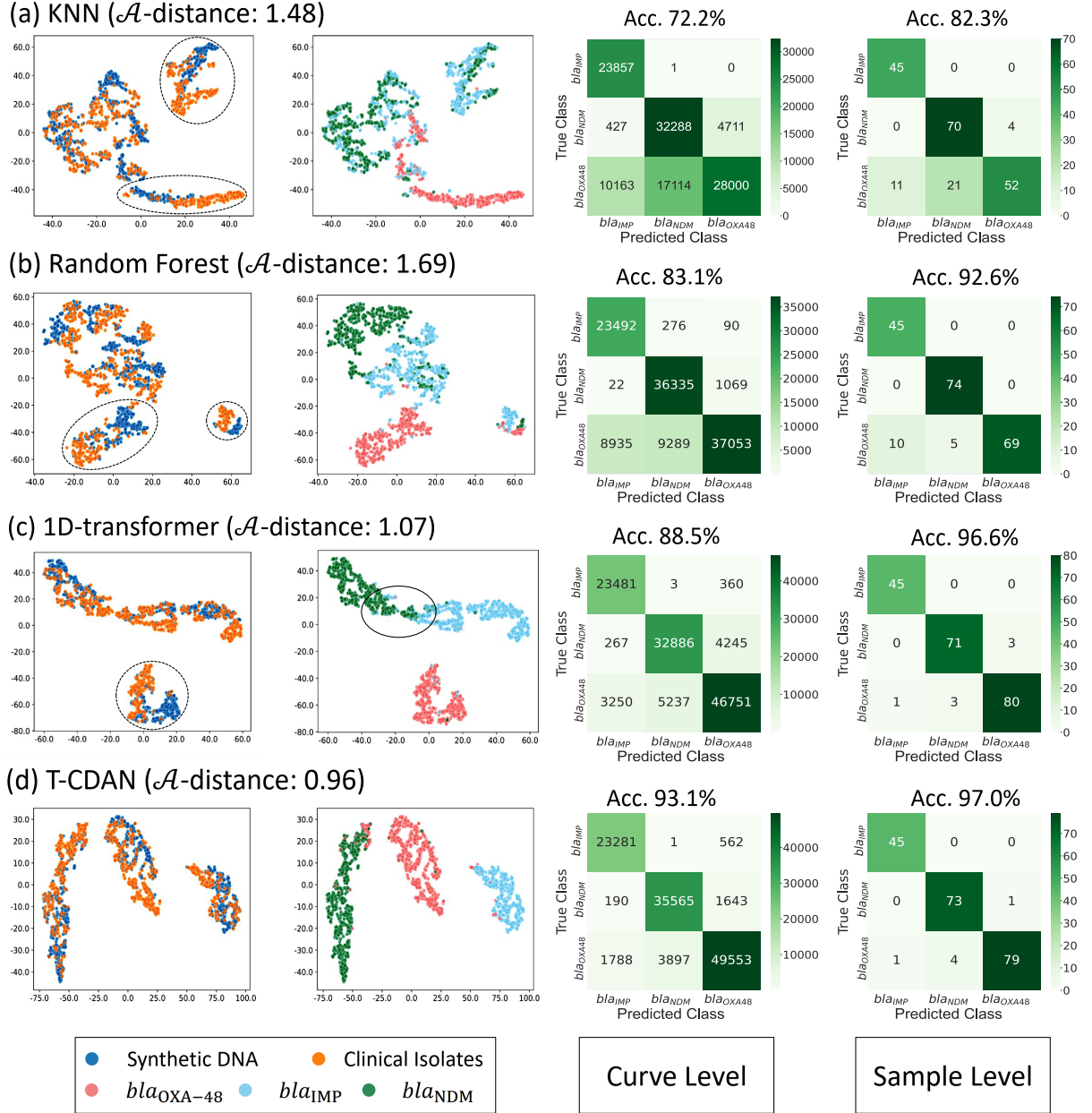


Figure 4.5: Visualisation and results of the considered methods. Each subtitle depicts the name of the evaluated methods and their related $\mathcal{A}$ -distance. Column 1&2: t-SNE feature visualisations, where features from different domains are coloured differently in Column 1 plots, and features from distinct AMR targets are coloured differently in Column 2 plots. Column 3: Curve-level classification confusion matrix. Column 4: Sample-level classification confusion matrix.

The target-labelled plots in Figure 4.5(a)-(d) (column 2) illustrate the distances of target clusters in the feature space, where more separate clusters with clearer boundaries and larger distances usually indicate easier classification. By using hand-crafted sigmoidal fitting parameters as features, the RF method outperforms the original KNN method with more separate target clusters, but there is still an isolated cluster that mixes all targets together. The

deep features extracted automatically by the transformer further reduce the overlapping area by enlarging the inter-target distances. Even though, cluster boundaries are still unclear between bla_{NDM} and bla_{IMP} features for the 1D-transformer, with a joint circle on the plot. By finding a common space for both domains, as shown in the second column of Figure 4.5(d), T-CDAN further benefits the target clustering and enables much clearer boundaries, with no overlap and only a tiny number of data points being clustered to the wrong group.

In addition, it is observed that T-CDAN features achieve the smallest $\mathcal{A}$ -distance among all models' features, implying that T-CDAN is the most effective method to reduce the domain gap. The 1D-transformer, with the second smallest $\mathcal{A}$ -distance, outperforms the RF and KNN methods in terms of reducing domain shifts, thanks to its ability of extracting features from higher-dimensional space compared to statistical learning-based methods. This outcome supports the inference we made based on the t-SNE feature visualisations.

4.4.3 Quantitative Evaluations

This subsection further analyses the four methods' quantitative results of target identifications. Confusion matrices at the curve and sample level for the four compared algorithms are illustrated in the last two columns of Figure 4.5. Although all the algorithms, to various extents, struggle in distinguishing bla_{OXA-48} , transformer-based approaches defeat the other two classical methods, with much fewer misclassifications shown for this target. The 1D-transformer presents slightly worse performance for bla_{NDM} than the RF method, but these drawbacks are partially eliminated by integrating the transformer into the CDAN framework. When considering sample-level results, the advantages of the newly proposed algorithms in clinical use are emphasised, with only 7 and 6 out of 198 samples misclassified for 1D-transformer and T-CDAN, respectively, compared to 38 for KNN and 15 for RF.

Detailed numerical results are shown in Table 4.1 and Table 4.2. For either curve or sample level, an increasing trend in both F1-score and Accuracy from KNN to T-CDAN methods for all targets can be observed (except for the F1-score between RF and 1D-transformer for bla_{NDM} , where the results of the new method slightly dropped), indicating a better overall performance when utilising the new strategies. Compared to KNN and RF, the transformer increases the mean accuracy by 16.3% and 5.4% at the curve level, and by 14.3% and 4% at the sample level. After applying the domain adaptation, T-CDAN further widens the performance gap by 20.9% and 10% at the curve level, 14.7% and 4.4% at the sample level. Providing the 97% of sample-level accuracy of T-CDAN, it is promising for this algorithm to be introduced to real-world clinical diagnosis after certain optimisations [159]. Although KNN and RF-based methods

show slightly higher sensitivity and specificity for some targets, their performance is severely biased and unbalanced among targets.

Table 4.1: Curve-level performance of four methods in three AMR target classifications.

		KNN	Random Forest	1D-transformer	T-CDAN
<i>bla</i> _{IMP}	Specificity (%)	69.3	72.4	87.0	92.2
	Sensitivity (%)	99.9	98.5	98.5	97.6
	F1-Score	0.818	0.834	0.924	0.948
<i>bla</i> _{NDM}	Specificity (%)	65.4	79.2	86.3	90.1
	Sensitivity (%)	86.3	97.1	87.9	95.1
	F1-Score	0.744	0.872	0.870	0.925
<i>bla</i> _{OXA-48}	Specificity (%)	85.6	97.0	91.0	95.7
	Sensitivity (%)	50.7	67.0	84.6	89.7
	F1-Score	0.636	0.793	0.878	0.926
	Mean Accuracy (%)	72.2	83.1	88.5	93.1

Table 4.2: Sample-level performance of four methods in three AMR target classifications.

		KNN	Random Forest	1D-transformer	T-CDAN
<i>bla</i> _{IMP}	Specificity (%)	80.4	81.8	97.8	97.8
	Sensitivity (%)	100.0	100.0	100.0	100.0
	F1-Score	0.891	0.900	0.989	0.989
<i>bla</i> _{NDM}	Specificity (%)	76.9	93.7	95.9	94.8
	Sensitivity (%)	94.6	100.0	95.9	98.7
	F1-Score	0.848	0.967	0.959	0.967
<i>bla</i> _{OXA-48}	Specificity (%)	92.9	100.0	96.4	98.8
	Sensitivity (%)	61.9	82.1	95.2	94.0
	F1-Score	0.743	0.902	0.958	0.963
	Mean Accuracy (%)	82.3	92.6	96.6	97.0

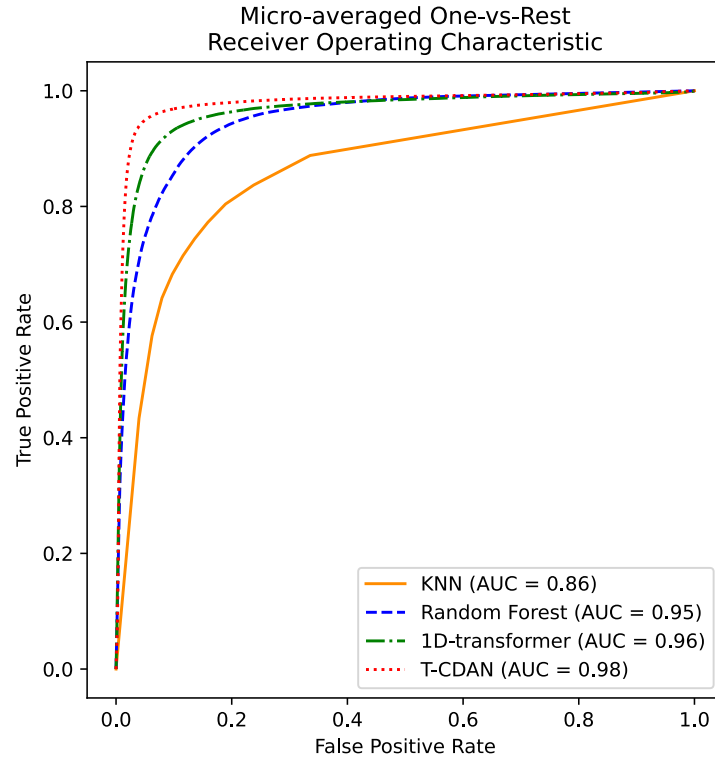


Figure 4.6: Micro-averaged ROC of all the compared algorithms.

Figure 4.6 shows the micro-averaged Receiver Operating Characteristics (ROC) of all the algorithms, illustrating the diagnostic ability of the classifier by plotting the true positive rate (sensitivity) against the false positive rate ($1 - \text{specificity}$), in which an Area under Curve (AUC) > 0.8 is usually considered a minimum threshold for clinical diagnostic tools. It is depicted that the proposed T-CDAN provides the highest AUC among all the compared methods.

4.5 Discussion

It can be seen from the findings presented in this chapter that transformer models extract discriminative features that are more robust to domain shift than other machine learning-based methods. The primary reason is that the positional encoding module enables the transformer to extract additional positional information from the curve. Furthermore, unlike other types of time series, real-time PCR amplification data from different targets represent a complex classification problem because of difficulties in manipulating the shape of this biological signal. As a result, the local features retrieved from the original dimensional space may be insufficiently discriminative. The encoding block in the transformer model can extract deeper features by mapping curves into a higher-dimensional space. In addition, providing that the

amplification curve can be further divided into four phases, the kinetic information from different phases can be effectively captured and aggregated by the multi-head attention mechanism of the encoding block.

T-CDAN outperforms all other methods in the AMR target classification because non-domain-adaptation-based methods only aim to perform well on the source domain data, whereas T-CDAN also ensures that the feature distributions across the two domains are as similar as possible. Besides, T-CDAN exploits multilinear mapping to control the uncertainty of label predictions, which guarantees the features' transferability and discriminability simultaneously. In addition, T-CDAN is also robust in the convergence, which is illustrated in Figure C.1.

Unlike conventional machine learning methods, the proposed T-CDAN provides balanced performance for all the target categories, which will benefit the real-world clinical application where observations of different pathogens follow various distributions [160].

This chapter demonstrated the proposed algorithm on qdPCR data. An apparent advantage of using dPCR for learning-based multiplexing is that numerous amplification reactions that happen simultaneously generate millions of amplification curves in a short period of time, providing the huge training dataset required by deep neural networks. Leveraging dPCR, we can further extend the training size and introduce more variability in our future work.

Like all other deep learning models, T-CDAN can be deployed on standard PC or workstations after the training process is finished, and the inference time for classifying new samples can be negligible, since the network size of the transformer backbone is not large. These facts make the proposed algorithm accessible to every PCR lab with basic computing resources.

There is also a demand for implementing the proposed deep ACA algorithm in conventional qPCR devices, which will widen the application scenarios and increase the clinical value of the methodology by integrating it into the current diagnosis flow without any hardware modification. Understandably, data from dPCR and qPCR will show heterogeneous structures with inconsistent distributions. The potential performance gap when transferring a dPCR-trained network to a qPCR application can also be filled by the proposed deep domain adaptation approach, inside which the qPCR data are considered coming from the target domain. Future work will focus on validating the T-CDAN on qPCR, also leveraging benefits of the large-scale dPCR training data.

Under certain circumstances, the data distributions shift among different experiments due to the variance of chemical agents, operation procedures, and manufacturer bias, which can become a significant factor causing the inter-experiment discrepancy in performance. If data

from each experiment are considered belonging to a separate domain, this issue can be described as a multi-domain adaptation problem. Future work will further extend T-CDAN into the multi-source one-target (multiple training experiments vs one test) and one-source multi-target (one training experiment vs multiple tests) manners, by integrating with other techniques such as knowledge distillation to learn domain-invariant features on multiple domains.

This chapter demonstrated the precise classification of three AMR genes. The next step is to enlarge the target number in the multiplex assay while maintaining a high accuracy level. Furthermore, research will be conducted for co-amplification situations, for example, when double infection occurs and more than one target shows up in a single sample. Preliminary results suggest the possibility of identifying co-infections by including them as additional categories and applying ACA accordingly [19], [69].

Regarding the economic benefit of single-well multiplexing, it can be assumed that by applying multiplex and multi-target detection of N targets in a single well, the total cost of screening these N targets would be reduced to $1/N$ compared to the traditional singleplex setting, which requires N times of reaction chambers. In addition, preparation time can be reduced, resulting in a simplified and lower workload for the entire testing.

4.6 Conclusion

This chapter proposed a novel framework, referred to as a Transformer-based Conditional Domain Adversarial Network (T-CDAN), to address the problem of domain discrepancy in ACA. This work is the first to incorporate the idea of a one-dimensional Transformer and CDAN to alleviate domain shifts in time series. Extensive experiments validated the effectiveness of the T-CDAN for the target identification of three AMR genes. A comparison of experimental results with the state-of-the-art ACA methods illustrates that T-CDAN achieves the most promising classification performance, by learning domain-invariant and discriminative feature representations from labelled synthetic DNA data and unlabelled clinical isolates data. T-CDAN provides the lowest $\mathcal{A}$ -distance value of all approaches and exhibits the clearest decision boundary between different targets in feature visualisations, indicating that it can significantly reduce the discrepancy between feature distributions from both domains while ensuring feature discriminability. T-CDAN's impressive performance in bridging the domain gap between multiple amplification curve datasets demonstrates its potential to address various types of domain shifts that may occur during NAAT multiplexing.

Chapter 5 AI-Driven Automated Colorimetric LAMP

5.1 Chapter Overview

The last three chapters discuss the contributions of this thesis in the data processing, assay design selection, and domain adaptive deep classification of ACA. In this chapter, the use of advanced signal processing and machine learning technologies is further extended to automated cLAMP, another application of data-driven approaches in molecular diagnostics, in addition to ACA. Portable POC devices have shown significant potential for rapid pathogen identification in limited-resource settings. cLAMP-based POC nucleic acid testing is particularly promising due to its speed, sensitivity, and simple visual readout. However, there is a need for a universal, automated colour detection method to improve ease of use and reduce readout subjectivity using portable devices. This chapter proposed an AI-driven workflow for automatic cLAMP-based multiple pathogen detection using a multi-tube strip format. Following sample extraction, reagent rehydration, and reaction incubation, the cLAMP panel was photographed with a portable device. The algorithm performed: (1) image pre-processing to locate tubes; (2) segmentation and cleaning to extract the liquid-filled region using deep learning and Otsu's Binarisation, followed by pixel cleaning; (3) empty tube and invalid control checks; and (4) colour detection using relative Maximum Mean Discrepancy to identify samples. The AI workflow was evaluated on 1,636 photos from 527 tests for the differential diagnosis of three panels, for skin-tropic viruses, respiratory viral pathogens, and malaria parasites, under various lighting conditions in POC environments. It was compared to human-labelled readouts, achieving 98.33%, 98.29%, and 96.68% accuracy in invalid photo rejection for the three panels, respectively, where all the misclassified images were caused by human mistakes or extreme lighting conditions, and 99.97%, 100%, and 99.69% accuracy in colour identification on accepted images. This chapter demonstrates a reliable automatic colour readout workflow for cLAMP-based nucleic acid detection, showing potential for integration into POC testing apps for cloud-based use.

The concept and experimental work presented in this chapter resulted in the following article under review:

K. Xu, M. Cavuto, K. Malpartida-Cardenas, M. Pond, D. Robinson, F. Kiemde, B. Hernandez, S. Sriskandan, U. D'Alessandro, A. Erhart, H. Tinto, A. Cunningham, A. Holmes, P. Georgiou, N. Moser, J. Rodriguez-Manzano, 'AI-Driven Workflow for Automated Colorimetric Isothermal Amplification for Robust Pathogen Detection on Portable Devices', under review. <http://dx.doi.org/10.2139/ssrn.4975777>

Contribution: methodology, software, validation, formal analysis, investigation, data curation, writing-original draft, writing-review & editing, visualisation. I served as the primary researcher for this study, independently handling all data science-related work, including but not limited to algorithm development, coding, data validation, and result analysis. I also conducted some parts of the wet-lab experiments.

5.2 Introduction

Diagnostics play a vital role in healthcare, yet a recent report from the Lancet Commission highlights that 47% of the global population still lacks access to essential testing [161]. This underscores the urgent need for more affordable diagnostic solutions. Lateral flow tests (LFTs) have gained widespread use due to their simplicity, low cost, accessibility, and rapid results, though they generally offer lower sensitivity compared to molecular methods like PCR, the current gold standard [162]. However, recent advances in portable molecular diagnostics have demonstrated great potential to deliver laboratory-grade sensitivity and specificity at the point of care, whether in GP clinics, homes, or resource-limited settings such as rural health facilities [163].

Among existing detection techniques for POC NAAT, LAMP has attracted particular interest because of its fast time-to-result, high sensitivity, and isothermal incubation requirements, which allow for a move away from bulky and expensive thermocycling instrumentation [53]. However, LAMP-based tests often rely on complex optical equipment for fluorescent-based readouts, limiting their portability. cLAMP simplifies this by using visual colour changes, allowing the detection of nucleic acid amplification based on pH-sensitive or metal ion indicators [23]. This method has been proven effective for detecting pathogens such as SARS-CoV-2 [26], [58], [105], [108], [164], influenza A and other respiratory viruses [28], *Plasmodium falciparum* [29], *Aspergillus fumigatus* [30], *Campylobacter coli* [31], *Streptococcus agalactiae* [32], *S. mansoni* and *S. haematobium* [27], HCV [33], HIV and HPV [34], unlocking an easy-to-use, affordable, and simplified alternative technology to pathogen screening.

Despite its advantages, the reliance on naked-eye readouts for cLAMP presents new challenges. Human interpretation introduces subjectivity and errors, particularly under poor lighting conditions, in instances of colour blindness or deficiency (which occurs in about 8% of men and 0.4% of women in European Caucasians [35]), or when colour differences are subtle or ambiguous [36], [37], leading to low efficacy and precision of epidemiologic investigations [31], [58], [108]. Leveraging standard camera technology on tablets or smartphones offers a low-cost solution to improve sensitivity and specificity in spotting positive samples, especially in situations of low initial target concentration and insufficient incubation. It also helps standardise automatic readouts to ensure reproducibility and facilitate multi-site collaborations. In addition, certain camera-based technologies enable automatic colour detection and target identification workflows, allowing for cloud-based automatic result reporting and integration into digital healthcare systems [59], [165].

However, automating cLAMP readouts presents its own challenges, mainly due to complex environmental factors such as ambient light conditions and dependency on device hardware [58]. Current efforts focus on three approaches: (1) custom-designed hardware platforms with built-in cameras/colour sensors, usually combining the heating unit for incubation with the colour readout units, which can either be built-in cameras [105] or dedicated colour spectrum sensors [27], [28], [32]. The customised aspect of the approach (fixed sensor specifications and controlled lighting conditions in the enclosed reaction chamber) allows to largely mitigate challenges in accurate colour interpretation. However, large hardware costs detract from the use of the approach at the POC, particularly in limited-resource settings. (2) Portable devices (e.g., smartphones or tablets) leveraged in controlled environments [26], [30], [31], [33], [58], [106], [107], with either an observation point for direct observation with the camera chamber [31], [106], [107], or an external component to repeatably align the camera with the sample tubes [26], [58]. In certain conditions where lighting and tube location are controlled, colour detection can be tackled with conventional threshold-based image processing algorithms [30], [31], [107], or radiometric indexes [29], [33], which only work for limited platforms. (3) Portable devices used in non-controlled environments that rely on advanced processing algorithms instead of specific hardware setups [108], [109]. Challenges like variable tube positions, shadows, overexposures, and inconsistent ambient lighting can significantly affect image segmentations and perceived colour values in the RGB space [107], invalidating traditional colour thresholding and identification methods. Deep learning has been introduced to address these challenges, but existing works often require extensive training datasets that

only work for the specific application scenario [108], [109], and raise concerns about interpretability and regulatory approval [110], [111].

To overcome these limitations, this chapter proposes a fully automatic cLAMP readout workflow with hybrid tube segmentation and colour detection algorithms. It is validated with Dragonfly, a rapid, portable, and easy-to-use POC cLAMP-based molecular diagnostic platform, developed by our group [166] and commercialised by ProtonDx Ltd. [167]. The Dragonfly platform offers several key advantages for point-of-care (POC) diagnostics: it enables rapid, multi-pathogen detection of pathogens in under 40 minutes, using a portable, instrument-light design that requires only an isothermal heat block. The system features power-free nucleic acid extraction and lyophilised colorimetric LAMP, eliminating the need for cold-chain storage and complex instrumentation. Overall, it minimises hands-on time and maximises accessibility in low-resource or field settings. The platform delivers accurate POC molecular diagnostics to resource-limited settings, simultaneously detecting and differentiating up to six distinct pathogen targets. However, the current platform relies on human interpretation for colorimetric readouts, offering opportunities to improve consistency and repeatability, as described above.

Dragonfly is paired with a companion app on a tablet, which is used to capture a photograph of the endpoint reaction tubes slotted in a custom card. The proposed AI-driven detection algorithms are applied to this photo following four steps: (1) image pre-processing: detection of card corners and identification of tube positions; 2) tube segmentation and pixel cleaning: enhancement of tube images with the Canny edge detector, followed by a combination of a state-of-the-art pre-trained deep segmentation model and adaptive colour thresholding to apply a context-based and transparency-based Region-of-Interest (ROI) recognition, and the recursive use of outlier detection-based unsupervised pixel cleaning; 3) tube check: exclude photos with invalid controls and empty tubes from further analysis; 4) colour detection: leveraging relative Maximum Mean Discrepancy (MMD) distance-based colour detection on ‘*H*’ channel to adaptively identify positive and negative samples.

Compared with previous research, the proposed algorithms have three innovations and advantages: 1) they do not rely on additional bulky and expensive hardware which increases the complexity of the setup and compromises the test’s portability; 2) no training data are required for the context (coarse) segmentation and following steps, thus avoiding generalisation issues; 3) the algorithmic workflow and controllable decision coefficient enable robust risk-free detection, matching medical device registration requirements.

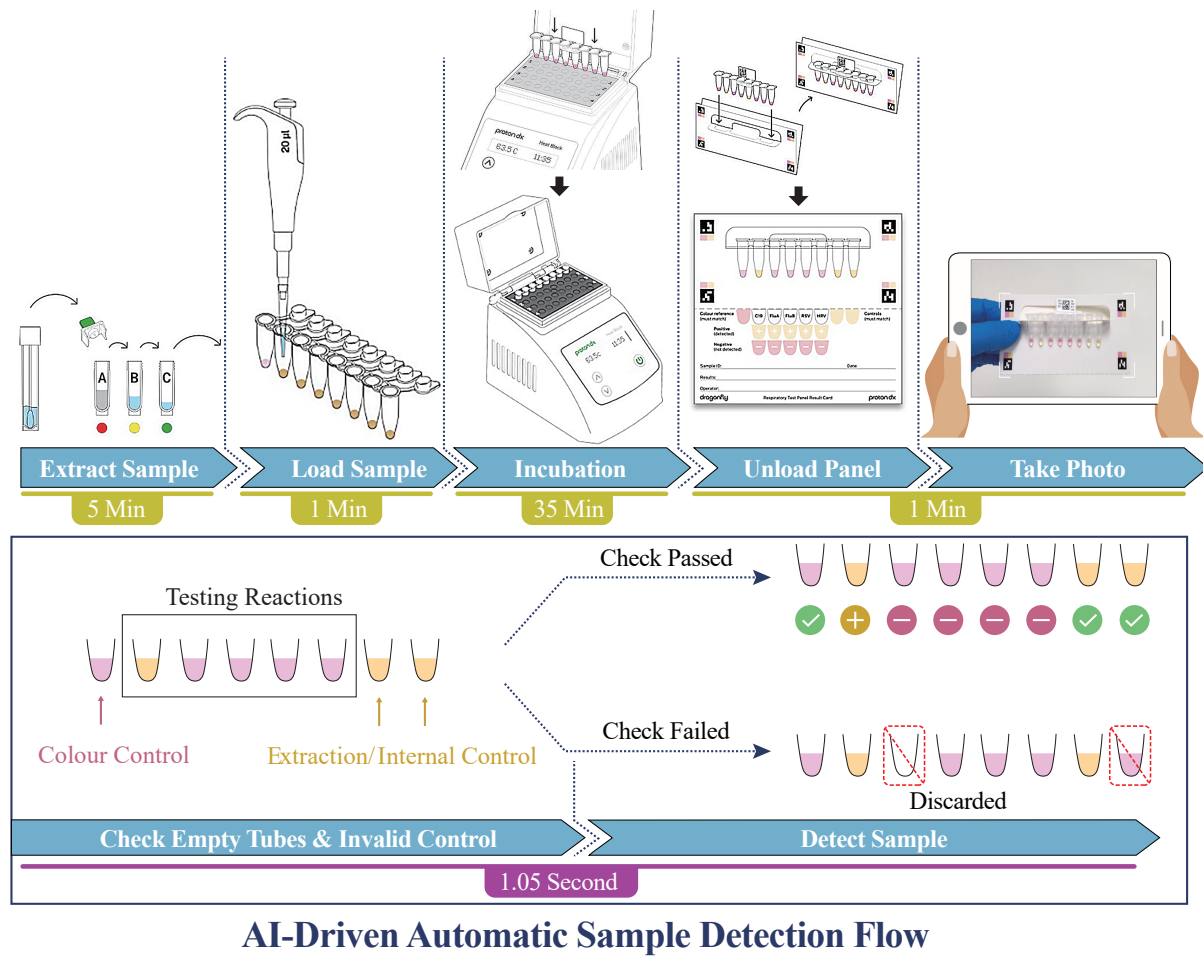
To evaluate the performance of the proposed workflow under different situations, three Dragonfly panels for different pathogens were used, generating three independent datasets. Dataset 1 includes 67 tests with the Dragonfly Skin Infection Viral Test Panel (subset of [166]), and Dataset 2 includes 129 tests using the Dragonfly Respiratory Test Panel (the panel presented in [167]). A total of 660 and 645 pictures were taken, respectively, for the two datasets using various portable devices, under different ambient lighting conditions. In addition, Dataset 3 is included, with 331 pictures taken during malaria screening at the community level across four rural villages in Burkina Faso, using multiple devices and involving multiple users, in uncontrolled lighting conditions. Highly reliable performance was achieved, with invalid photo rejection accuracy of 98.33%, 98.29%, and 96.68% respectively, for the three panels, and 99.97%, 100%, and 99.69% accuracy of colour identification. This chapter demonstrated a reliable automatic colour readout flow for vast applications in cLAMP-based pathogen detections for infectious diseases, which could also be generalised for other colorimetric amplification chemistries. The approach holds significant potential for integration into POC testing apps for cloud-based diagnostic flows.

5.3 Material & Methods

5.3.1 The Dragonfly Platform

Dragonfly Testing Kit, developed by Dr Rodriguez-Manzano's laboratory and now commercially available by ProtonDx Ltd., UK, an Imperial College Startup, is a portable, sample-to-result molecular diagnostic platform designed for rapid multi-pathogen detection. Combining a simple power-free nucleic acid extraction method based on magnetic beads [168] and lyophilised cLAMP technology [169], Dragonfly enables a rapid diagnostic solution without needing thermocycling, fluorescent readout, or cold-chain storage.

The algorithms described in this chapter are aimed at being integrated into the Dragonfly Kit, providing an automatic solution from photo taking towards result report generation. The details of the Dragonfly Kit are depicted in Figure D.1, which includes a sample preparation kit, a test panel, a heating block, and the user app. Although developed on Dragonfly, the proposed algorithm workflow could be adapted for other diagnosis platforms and/or with other amplification chemistries.



AI-Driven Automatic Sample Detection Flow

Figure 5.1: Dragonfly workflow (adapted from [167]) with the proposed AI-driven readout flow. Samples are (1) extracted with magnetic bead-based extraction kits, and (2) loaded into an 8-tube panel. The first and the last two tubes function as colour and extraction/internal controls, and Tube 2-6 are for pathogens. The panel is then (3) inserted inside a dry bath heat block, and incubated for 25 minute at 63.5°C. After the incubation, the panel is unloaded and (4) placed inside a Result Capture Card. (5) A tablet/smartphone app takes a photo of the card, to which the proposed AI-driven detection algorithms are applied. (6) Empty tube and control checks are then performed to exclude invalid panels. (7) Colour identification runs on check-passed panels, outputting the detected colour/testing result of each testing reaction tube.

As depicted in Figure 5.1, after sample extraction, DNA/RNA is isolated, purified, and concentrated through the SmartLid method [167], [170], and the eluted nucleic acids are loaded into a panel of eight tubes, resuspending lyophilised cLAMP reagents. The first tube acts as a colour control and is designed to always remain pink (negative), through the exclusion of critical amplification enzymes. The last two reactions function as the extraction and internal controls, respectively, and should always be yellow (positive) in the case of a valid test result. Tube 2-6 are the testing tubes and contain cLAMP assays that target five distinct pathogens and indicate the diagnostic result in the case of valid control reactions. The panel is further incubated,

unloaded, and placed into a Result Capture Card, which has a panel holder and four AprilTags in the corners [171]. Chemical details of Dragonfly are further explained elsewhere [166].

An integrated app, running on the portable device, guides the users through the testing process and captures a photo of the incubated panel placed on the Result Capture Card. After tube colours are identified, the final testing results are uploaded securely to the Cloud platform. There is no specific requirement for the device running the app, as long as a camera with >5MP resolution is provided.

5.3.2 Datasets in This Chapter

For validation purposes, the proposed algorithm flow was demonstrated on three test panels: respiratory viral pathogens (for SARS-CoV-2, influenza A virus, influenza B virus, respiratory syncytial virus, and human rhinovirus), skin-tropic viruses (for orthopoxvirus, monkeypox virus, varicella zoster virus, human herpesvirus 1, human herpesvirus 2), and the malaria panel. Examples of tests from the three panels are depicted in Figure 5.2.

Each experiment is labelled as positive (at least one positive reaction), negative (all testing reactions negative), empty (at least one tube is empty due to improperly loaded/placed stripes), or invalid control (at least one control colour is incorrect due to operation errors such as sample not loaded or incorrect sample extraction).

For the first two panels, two datasets were randomly selected: 67 tests using the Skin Infection Viral Test Panel [166] (33 positive, 28 negative, 1 empty, 5 invalid control) and 129 tests with the Respiratory Test Panel [167] using spiked samples (52 positive, 58 negative, 7 empty, 12 invalid control). Each test panel was photographed 5 to 10 times using the built-in cameras of an iPhone 12 (12 MP) and an iPad Gen5 (8 MP) (Apple Inc., US). To evaluate the robustness of the algorithm flow under various lighting conditions, photos were taken in a standard PCR lab with white ceiling lights, with each panel held vertically around 20 cm, 40 cm, 60 cm, and 100 cm from the source, and 1-2 photos taken at each position. This results in 660 and 645 pictures for Dataset 1 and 2, respectively. An example picture captured is shown in Figure 5.3 “Raw Photo”. All the photos were labelled by human naked-eye readouts with expert check as the ground truth for evaluation (not used for training).

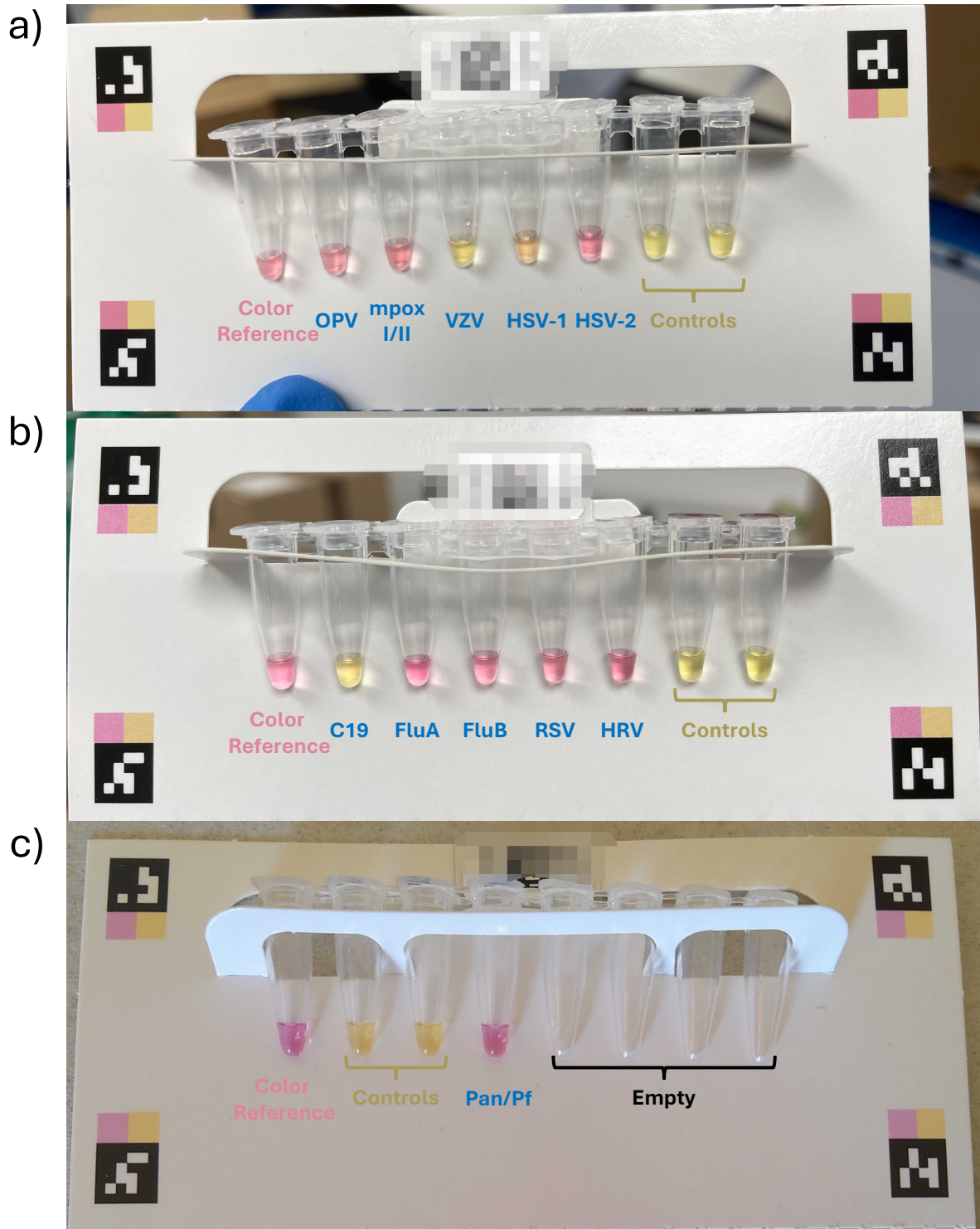


Figure 5.2: Examples of sample results from a) Skin Virus Panel, including orthopoxvirus (OPXV), monkeypox virus (mpox I/II), varicella zoster virus (VZV), human herpesvirus 1 (HSV-1), and human herpesvirus 2 (HSV-2); b) respiratory panel, including SARS-CoV-2 (C19), influenza A virus (FluA), influenza B virus (FluB), respiratory syncytial virus (RSV), and human rhinovirus (HRV); and c) malaria panel for detection pan-malaria. For a) and b), colour reference and controls are distributed in the first and the last two positions, with Tube 2-6 as tests. c) follows a different pattern in which colour reference and controls lie in the first three positions, the test tube is located in the fourth, and the rest remain empty. Adaptations were carried out to apply the proposed algorithm flows to the malaria panel.

For the malaria panel, 331 blood samples from asymptomatic individuals were tested and taken photos on-site (outside health facilities, i.e., directly at community level), across four different rural villages in Burkina Faso. by different operators from 25 Nov 2024 to 12 Dec 2024, using the built-in camera of Samsung Galaxy Tab S5e (13MP, Samsung Electronics Co., Ltd.). No standardised guidelines were provided to the operators for capturing the photos, aside from the use of the Result Capture Card. This dataset reflects the real-world situations of POC applications in the field, where challenging lighting conditions and operation variance exist. Since only one target pathogen is tested in this panel and reference tubes are reallocated, the following algorithm flow was adjusted accordingly when applied to this dataset.

5.3.3 Algorithm Flow for Automatic Colour Detection and Target Identification

The algorithm flow processes the raw photos captured by the Dragonfly App, delivering pathogen detection results or rejecting photos based on empty tubes or invalid controls. Figure 5.3 depicts the algorithm flow we proposed, which contains four parts: image pre-processing, tube segmentation and pixel cleaning, tube check, and colour detection. All the algorithms were developed and evaluated on a workstation (CPU Intel Xeon Gold 6226R * 2, RAM 128GB, GPU NVIDIA RTX A5000) with Python 3.9.17. Codes and a demonstration dataset are available elsewhere [172].

5.3.3.1 Image Pre-Processing

The user holds the Result Capture Card in front of the camera, which captures the raw image if at least three AprilTags are detected. AprilTags are special black-and-white markers originally designed for robot applications, enabling fast target location identification [171]. Step 2 involves detecting the four corners of the card based on the identified AprilTag locations, applying trapezoidal rectification, and resizing the image. After this step, the card is cropped into a fixed size of 2000*500 pixels.

The Result Capture Card is designed to hold the tubes at a constant X-axis position, while there may be Y-axis variations depending on user operations. Therefore, Step 3 crops the approximate location of the tubes by dividing the designated X-axis locations of the card while keeping a relatively large Y-axis range. In most cases, after this step, each cropped image will contain one tube and surrounding background pixels, making it ready for tube segmentation in the next step.

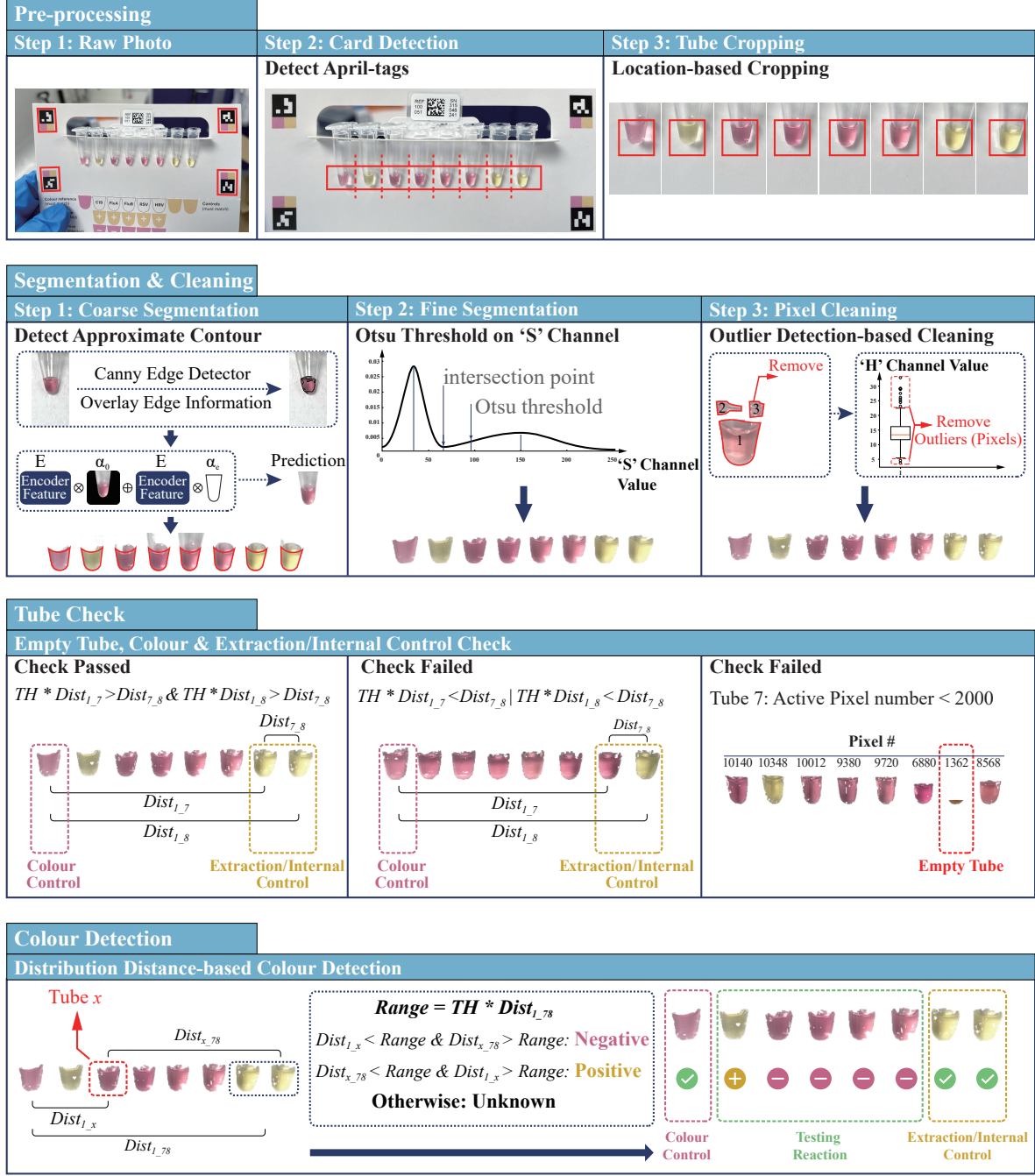


Figure 5.3: Proposed algorithm workflow. Pre-processing: Step 1 inputs a raw photo; Step 2 detects and adjusts the card location based on the AprilTags in the four corners; Step 3 crops the approximate images of tubes based on relative locations. Segmentation & Cleaning: Step 1 enhances the image with the Canny edge detector, and coarsely segments the tube area using a pre-trained TRACER model; Step 2 finely segments out the solution-filled area by applying the Otsu's binarisation on the 'S' channel of coarse segmented tube images; Step 3 recursively cleans the fine segmented images utilising Connected Component Analysis, K-means clustering, and the Random Forest-based outlier removal algorithm. Tube Check: check the existence of empty tubes with active pixel numbers and the correctness of control colours with MMD-based relative colour distance comparison. Colour Detection: panels that pass the previous check are adopted with colour detection by comparing the to-be-detected colour with both negative and positive control colours and choosing the more similar one as output, under the scheme of relative MMD distance measuring of 'H' values.

5.3.3.2 Tube Segmentation and Pixel Cleaning

Although an approximate location of the tube is known from the previous step, it is not practical to use all the pixels of the tube image and classify colour values, since the background pixels may contain shadows and light spots which can affect colour value distributions [107]. Furthermore, certain pixels in the solution-filled area may be influenced by reflections under extreme lighting situations. Therefore, the fine contour of the solution-filled area of the tube (tube tip) needs to be depicted, and the tube tip needs to be segmented and cleaned. To achieve this goal, three steps are involved.

Firstly, the tube must be segmented from the background (coarse segmentation). Since the textural features of the background are complex and conventional colour-based thresholding may not work properly, we adopted a state-of-the-art deep learning model, TRACER_B7, which has been pre-trained on large natural image datasets [173], [174] to identify the tube as an object. Before inputting the tube image into the network, a Canny Detector with threshold 1 as 15 and threshold 2 as 30 was applied and added to the picture to emphasise the edge information and mitigate the impact of out-of-focus blur on object contour, generating the enhanced pictures [175]. The TRACER detects objects using attention-guided tracing modules incorporated with explicit edges [173]. With enhanced inputs, this pre-trained model enables precise tube identification without the need for additional data labelling, thereby facilitating the transition to new scenarios and avoiding issues with generalisation.

Secondly, the TRACER model can roughly segment the tube from the background, yet the output of the network may contain both the empty and the solution-filled parts of the tube. Also, the segmentation details can be affected by ambient factors such as reflections. Therefore, fine segmentation is required as the second step to further narrow the ROI into merely the tube tips, where pixel colours are pure. For colour-based operations, it has been proven that using the HSV space, which linearly represents the hue with only one value, can effectively eliminate the lighting-induced colour value variations when using commercial cameras in ambient environments [105]. Therefore, we first transformed the coarse-segmented image from RGB to HSV space [176]. We then used the ‘S’ channel value to represent the image as a grayscale picture, depicting the colour saturation. Provided the assumption that the empty area of the tube is more transparent and less saturated compared with the filled tip, Otsu’s binarisation was used to fine-segment the tube tips by finding a threshold k in the ‘S’ channel histogram which minimises the following weighted within-class variance [177]:

$$\sigma^2 = p_A(k)p_B(k)(m_A(k) - m_B(k))^2 \quad (5.1)$$

where

$$p_A(k) = \sum_{i=0}^k p_i, \quad p_B(k) = \sum_{i=k+1}^{255} p_i, \quad (5.2)$$

$$m_A(k) = \frac{\sum_{i=0}^k i p_i}{p_A(k)}, \quad m_B(k) = \frac{\sum_{i=k+1}^{255} i p_i}{p_B(k)}. \quad (5.3)$$

p_i is the probability of a pixel of grayscale value i , $p_A(k)$ and $p_B(k)$ are the probability of a pixel to be categorised into Group A (pixel value $< k$) and Group B (pixel value $> k$), $m_A(k)$ and $m_B(k)$ are the mean values of the two groups. The optimal k was found by applying a grid search in $[0, 255]$.

Finally, after applying the fine segmentation, pixel cleaning was adopted to remove the abnormal pixels passed from the previous steps, which may be caused by light spots or reflections on the tube wall. The detailed algorithm for pixel cleaning is illustrated in Algorithm 5.1 and Figure 5.3 Segmentation & Cleaning Step 3. The RGBA image was first transformed into HSVA space, following which a connected component analysis (CCA) [178] was introduced to collect the connected areas of active pixels (where transparency channel ‘A’ value = 255) and keep only the two largest connected components (e.g., in the figure the third largest component is removed). After that, recursive steps of K-means two-clustering on ‘S’ channel values and removing the cluster with the smaller ‘S’ channel median value were applied, until the total number of active pixels was smaller than 14,000 (average size of tube tips). Finally, an Isolation Forest-based Outlier removal algorithm [131] with a 20% contamination ratio was adopted on ‘H’ values of active pixels to remove the outlier pixels that have significant differences with the majority. After pixel cleaning, the remaining active pixels should be suitable for colour detection in the following steps.

Algorithm 5.1: Algorithm Flow for Pixel Cleaning.

Step	Input:	Comment
	R, G, B, A	RGBA image matrices
	Output:	
	A_{mask}	Masked A matrix with selected pixels as 1
1	$R, G, B, A \rightarrow H, S, V, A$	Transfer RGBA to HSVA space
2	CCA on active pixels ($a = 255$ in A)	
3	Descending rank N connected components	

4	If $N > 2$:	
5	Remove pixels ($a \leftarrow 0$) $\in$ Component 3, 4, ..., N	Keep only the first two components
6	End If	
7	While <i>number of $a=255$ in $A > 14,000$:</i>	Recurrent pixel cleaning
8	Cluster index c_1, c_2 $\leftarrow$ Apply k-means clustering on S values where pixel $a=255$ in A	
9	If $\text{median}(S_{c_1}) > \text{median}(S_{c_2})$:	
10	remove pixels $\in c_2$	
11	Else:	
12	remove pixels $\in c_1$	
13	End If	
14	End While	

5.3.3.3 Tube Check

Before identifying the solution colours, the algorithm must check whether empty tubes or invalid controls exist and decide whether the photo should be rejected. Empty tubes were picked out and discarded from further analysis if any tube showed an active pixel number smaller than 15% (around 2000 in this research) of the normal pixel number for a tube after pixel cleaning.

We then define the colour distance between Tube x and Tube y as:

$$Dist_{x,y} = MMD(\mathbf{H}_x, \mathbf{H}_y)^2, \quad (5.4)$$

$$MMD(\mathbf{H}_x, \mathbf{H}_y)^2 = \frac{1}{m^2} \sum_{i=1}^m \sum_{j=1}^m k(h_{x_i}, h_{x_j}) + \frac{1}{n^2} \sum_{i=1}^n \sum_{j=1}^n k(h_{y_i}, h_{y_j}) - \frac{2}{mn} \sum_{i=1}^m \sum_{j=1}^n k(h_{x_i}, h_{y_j}), \quad (5.5)$$

$$k(h_1, h_2) = \cos\left(\frac{\pi}{360} \cdot d(h_1, h_2)\right), \quad (5.6)$$

$$d(h_1, h_2) = \min(|h_1 - h_2|, 360 - |h_1 - h_2|), \quad (5.7)$$

where $\mathbf{H}_x = [h_{x_i}]_{i=1,\dots,m}$ and $\mathbf{H}_y = [h_{y_j}]_{j=1,\dots,n}$ are the active pixel column vectors of Tube x and y in H values. Following the concept of MMD [179] and considering the nature of HSV space as a cylindrical coordinate system, we defined a new kernel function $k(h_1, h_2)$, called

Period-Linear Kernel, which calculates the minimal angle on the circle of H values as the inter-colour distance $d(h_1, h_2)$ and maps it to the range $[0,1]$. Therefore, $Dist_{x,y}$ is a measurement of the H value distribution discrepancy between the two tubes. Smaller $Dist_{x,y}$ indicates more similar colours in tubes.

The following criteria are used to check whether control tubes show the correct colours:

$$TH * Dist_{1,7} > Dist_{7,8} \text{ and } TH * Dist_{1,8} > Dist_{7,8} \rightarrow \text{Check Passed} \quad (5.8)$$

$$TH * Dist_{1,7} < Dist_{7,8} \text{ or } TH * Dist_{1,8} < Dist_{7,8} \rightarrow \text{Check Failed} \quad (5.9)$$

where TH is an adjustable coefficient and is fixed at 0.3 in this chapter. As we mentioned before, Tube 1 serves as the colour control and should always be pink, whereas Tube 7 and 8 are Extraction and Internal Control and should always be yellow. The criteria require the distance between Tube 7 and 8 (which should be substantially small) to be much smaller than both the Tube 1-7 and Tube 1-8 distances (which should be substantially large). If any criterion is not satisfied, the algorithm considers the controls incorrect and rejects the photo. Instead of using fixed colour value ranges to define colours, the proposed criteria measure relative colour differences and make judgments based on the chemical nature of the reactions, to avoid the potential colour value shifts caused by lighting conditions or camera specification discrepancies.

5.3.3.4 Colour Detection

Images that successfully passed the tube check would be accepted for the final colour (target) detection. This section followed the same concept of relative colour distances in the detection step, setting the criteria below for Tube x :

$$Dist_{1,x} < Range \text{ and } Dist_{x,78} > Range \rightarrow \text{Negative} \quad (5.10)$$

$$Dist_{x,78} < Range \text{ and } Dist_{1,x} > Range \rightarrow \text{Positive} \quad (5.11)$$

$$\text{Otherwise} \rightarrow \text{Unknown} \quad (5.12)$$

where

$$Range = TH * Dist_{1,78} . \quad (5.13)$$

$Dist_{1,78}$ indicates the MMD distance between the H values of cleaned Tube 1 and the combined H values of cleaned Tube 7 and 8. To intuitively understand the formula, $Range$ can be considered as the distance between the colour Pink and colour Yellow (no fixed colour values are used) times a small coefficient. For a new tube (Tube x) to be detected, the algorithm

checks respectively its H values' distance with the standard colour Pink ($Dist_{1_x}$) and Yellow ($Dist_{x_{78}}$). If one of the two distances is smaller than $Range$ and the other one is not, Tube x must have a similar colour to the corresponding standard colour; thus, a decision can be made. Otherwise, Tube x is close to neither of the standard control colours, and an 'Unknown' result will be exported. By changing the coefficient TH , the strictness of the decision could be adjusted, enabling a controllable decision-making process, which is required for medicine-grade AI algorithms.

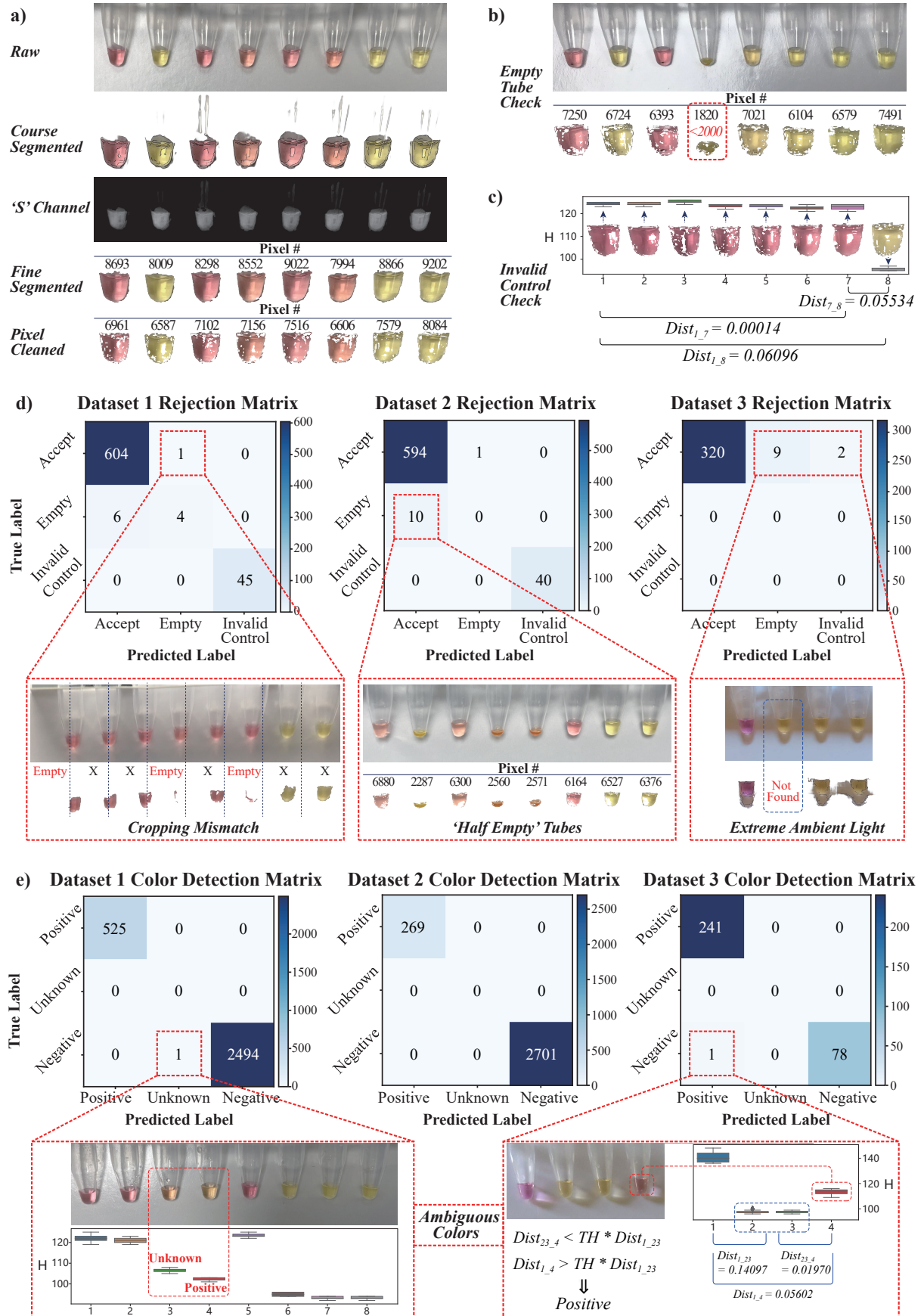
Once the proposed algorithm flow is integrated into the app, the detected results for the five pathogens can be presented to the user through the software, which will also give feedback and instructions on any of the exceptions (empty tubes, invalid controls), allowing the user to redo the experiment or retake the photo from the initial step.

5.4 Results

The qualitative and quantitative performance evaluations of the proposed algorithm flow are provided in Figure 5.4. Note that for the malaria panel, the colour, extraction and internal controls are located in Tube 1-3, respectively, and only one test is conducted in Tube 4, so the steps of Tube Check and Colour Detection were adapted accordingly following the same concept.

5.4.1 Examples of Tube Segmentation and Pixel Cleaning

A demonstration of tube segmentation and pixel cleaning is illustrated in Figure 5.4(a), where the raw image, the coarse-segmented tubes and their corresponding 'S' channel grayscale images, the fine-segmented tubes, and tubes after pixel cleaning are presented. The background of the raw image contains complex textural features due to the poor ambient lighting, but the TRACER network with enhanced inputs can still roughly extract tube locations and contours, providing smooth edges. However, since the model extracts context-based information, it also includes some transparent parts of the tubes affected by complex ambient lighting. Analysing the 'S' channel of the HSV space reveals significant differences between empty and filled tube areas, where solution-filled areas have higher 'S' values and look brighter. This creates a bimodal distribution for 'S' values of active pixels and benefits the further step of Otsu's binarisation. The Otsu's threshold successfully separates the background and ROI, outputting clean tube tips with almost no empty parts.



channel grayscale images, the fine segmented tubes, and tubes after pixel cleaning on each row, respectively. **b)** A panel with an empty tube (Tube 4), in which the active pixel number is smaller than the empirical threshold of 2000. **c)** An example where incorrect extraction control occurs, with boxplots of ‘*H*’ values shown. MMD distances $Dist_{1_7}$, $Dist_{1_8}$, and $Dist_{7_8}$ are presented. **d)** The rejection matrix of Dataset 1-3 depicting the quantitative performance of the empty & control check. Numbers in the matrices indicate the number of panel photos of the corresponding category. Three examples of incorrectly rejected photos are illustrated, in which a cropping mismatch, a ‘Half-empty’ issue, or extreme ambient lighting occurs. **e)** Confusion matrices of colour/target detection performance on accepted panels (panels that belong to the upper-left corners of rejection matrices) for Dataset 1-3, with misclassified samples presented, in which ambiguous colours exist.

After pixel cleaning, approximately 15%-20% of outliers with extreme ‘*H*’ values are removed, resulting in slightly fewer pixels and blank points in the ROI, as shown in Figure 5.4(a) Row 5. Although the tube tips may look ‘broken’ after pixel cleaning, the algorithm narrows the ‘*H*’ value distribution, purifying the colour hue, and making the following distance measurement more accurate.

5.4.2 Demonstrations of Empty Tubes and Invalid Controls

Figure 5.4(b) illustrates a panel with an empty tube, which can be caused either by operational errors or promote evaporation induced by long-time storage. As the active pixel number for Tube 4 is smaller than 15% of the normal pixel number for a tube (approximately 2000), this photo is rejected, and the user will be asked to re-run the experiment.

The rejection matrices of Dataset 1 & 2 show tubes considered empty by the ground truth are accepted by the algorithm. This is due to the ‘Half empty’ issue, as demonstrated in Figure 5.4(d), where the reactions are completed but there is less solution. This issue will not affect the colour detection accuracy since all the wrongly accepted tubes are identified as the correct colour. Further discussion can be found in 5.5.2.

5.4.3 Performance of Target Detection

Figure 5.4(e) provides the colour/target detection accuracy of the three datasets in confusion matrices. The numbers in the matrices indicate the numbers of testing reaction tubes of accepted photos (photos categorised into the top-left corners of rejection matrices). The proposed algorithm achieved 99.97%, 100%, and 99.69% accuracy of colour identification, respectively, with only two out of 6310 tubes misclassified. The two wrongly detected samples are presented in Figure 5.4(e) lower part, in both of which ambiguous colours exist. Boxplots also show ‘in between’ distributions of pixel colours. Further discussion is provided in 5.5.1.

5.5 Discussion

5.5.1 Sensitivity to Ambiguous Colours

As depicted in Figure 5.4(e), in some situations, tubes may exhibit ambiguous colours between pink and yellow, which can be unfinished reactions caused by insufficient incubation time. To explore the algorithm's response to this abnormal situation and evaluate the method's sensitivity, supplementary experiments were carried out using skin-tropic virus panels with three concentrations, taking photos every 5 minutes after incubation. The colour transforming phases were spotted, and the algorithm performed robustly with high sensitivity in abnormality detection when ambiguous colours showed up, as long as the TH is set inside a reasonable range. Technical details and results can be found in Figure D.2, Figure D.3, and Section D.2. This further emphasises the necessity of using an automated readout workflow, as it can be more sensitive and reliable in spotting positive samples, especially in situations of low initial target concentration and insufficient incubation.

5.5.2 Consequences of Misclassification on the Workflow

The 'Half-empty' issue occurs due to human errors or delayed panel storage. Delays between running the test and capturing the result may cause a shifted colour hue, and user handling errors may lead to reduced solution volume. Even under this extreme circumstance of 'half-empty', the proposed workflow can still correctly identify the target status, showing its reliable performance in real clinical scenarios.

Certain abnormalities may increase the rejection ratio. These abnormalities are either caused by human errors (such as cropping mismatch caused by incorrectly placed panels, and large bubbles in solution), or induced by extreme lighting conditions (e.g., shadow or dark zone in the picture). However, they will not influence the final detection accuracy, as these panels are rejected during tube check and excluded from further steps. After integrating the proposed algorithm flow in the Dragonfly App, when the tube check fails, user alerts could provide instructions like adjusting the camera angle, retaking photos, or replacing the panels. Future efforts will focus on enhancing the photo cropping process to handle various panel placements.

5.5.3 Importance of Using Relative Colour-Distancing

Although the H value is considered robust against lighting changes, variability in the production (e.g., lyophilisation process) or formulation (e.g., different batches of reagents) may

still affect the colour of positive/negative reactions, resulting in different H values among batches and among reactions. This further emphasises the necessity of utilising a relative colour-distancing system instead of threshold-based decision-making in colour identification. We envision vast applications of the proposed workflow, with its adaptive and relative decision strategy, in future scenarios of POC cLAMP diagnosis without the need for sophisticated adjustment, as demonstrated when applied to Dataset 3.

Thanks to its adaptive and training-free framework, the proposed algorithm flow, initially developed for cLAMP-based applications, can also be generalised for other colorimetric amplification chemistries such as colorimetric rolling circle amplification (RCA) and strand displacement amplification [57], as long as the contrasting colours of positive and negative references are provided.

5.5.4 Limitations and Future Work

The algorithm flow is intended to be embedded in a user app on portable devices, raising concerns about its computational efficiency, especially for the deep network part. However, the unsupervised nature of the workflow avoids computationally expensive data training, and inference can be efficiently handled by cloud-based GPU servers or AI edge computing on portable devices. Future efforts will focus on implementing the algorithms into portable devices with optimisation techniques such as network quantisation and compression to enable a seamless photo-to-result experience in real-world applications.

Although we tested the algorithm flow on thousands of reaction tubes taken under a variety of different lighting conditions, further validation in uncontrolled environments is necessary. Future work will focus on integrating the proposed methods into the Dragonfly App and validating the entire workflow across a broader range of environments to demonstrate its compatibility with fully uncontrolled environments, leading towards the implementation at the POC.

It is important to note that in this chapter, a unique form of multiplexing is employed for cLAMP, where each tube performs a singleplex reaction, as opposed to the single-well multiplexing used in other chapters. This approach is necessitated by the limitations of the current colorimetric technique, which allows only one target-specific colour change to be visually identified per well. However, the proposed AI-based workflow introduces a promising avenue for single-well multiplex cLAMP, in which the subtle colour differences during and at the end of reactions may be captured and interpreted.

5.6 Conclusion

This chapter proposed an automated cLAMP readout workflow for multiple pathogen identification. The efficacy of the method was verified on 1636 testing panel photos of 527 samples tested with the Dragonfly Kits, with variations of photo-taking devices and ambient lighting conditions. Evaluation results show over 96% accuracy in rejecting abnormal panel images with empty tubes or invalid controls, and over 99% accuracy in colour detection. This multistep detection workflow, leveraging hybrid ROI segmentation and relative distance-based adaptive colour identification, is unsupervised and can be applied to other cLAMP testing scenarios without significant modifications. The proposed algorithm flow may be integrated with emerging cloud-based POC techniques, providing a powerful tool for the surveillance of infectious diseases and epidemiological investigations.

Chapter 6 Time-to-Positive Modulation: Enhancing ACA in qLAMP

6.1 Chapter Overview

Previous chapters tackle the issues of AC data pre-processing, multiplex assay development, and cross-domain ACA with dedicated algorithms, validated on qPCR and qdPCR. However, as we discussed in 1.3.5, transferring these techniques directly to qLAMP includes additional challenges due to its sophisticated chemical nature. Unlike PCR, LAMP's continuous isothermal amplification and branched mechanism generate homogeneous amplification curves that challenge real-time target discrimination. While this uniformity enables rapid diagnostics, it limits the discriminative potential of fluorescence signals, necessitating endpoint or post-amplification strategies for target identification. This chapter explores a novel computational framework for enhancing multiplex detection using qLAMP. To overcome these challenges, this chapter introduces a method to modulate amplification curve characteristics by adjusting the relative concentrations of LAMP primers. Initial experiments reveal that while primer concentration does not predictably influence fluorescence intensity at saturation, it significantly affects the reaction TTP. Leveraging this finding, the chapter proposes TTP modulation as a strategy to separate targets into non-overlapping temporal windows based on primer concentration and detection threshold. A computational framework is developed to model the relationship between primer concentration, template concentration, and TTP, using a two-term power model. Primer concentrations are then optimised globally to minimise overlap in TTP ranges across targets. Where overlap remains, a binary ACA is employed to resolve classification ambiguities. This hybrid approach effectively transforms a multi-class identification problem into tractable binary decisions, enhancing accuracy and scalability. Empirical validation demonstrates the feasibility of the approach, highlighting its potential to simplify and automate qLAMP assay design for multiplex diagnostics.

Contribution: I served as the primary researcher for this study, independently handling all data science-related work, including but not limited to algorithm development, coding, data validation, and result analysis. I also conducted some parts of the wet-lab experiments.

6.2 Introduction

Compared with PCR, LAMP produces fluorescence curves that rise faster and appear almost indistinguishable across different targets. Two principal mechanistic factors explain this behaviour. First, LAMP is an isothermal process that proceeds continuously at a single temperature, typically between 60 °C and 67 °C, so the enzyme never idles during discrete denaturation, annealing, or extension phases [53], [54]. The strand-displacing Bst Polymerase binds a primer, elongates the strand, and simultaneously displaces downstream DNA in one uninterrupted motion. Eliminating the heat-cool transitions of conventional PCR removes a considerable amount of dead time: a qPCR thermal block may spend 15-20s per cycle merely climbing or falling to the next set-point, which translates to roughly half of the total run time. LAMP avoids this overhead entirely.

Second, each LAMP target is addressed by a set of four to six primers that introduce two loop regions into the product. Once a number of “dumbbell” molecules have formed, those structures fold back on themselves and self-prime recursively [54]. The reaction therefore branches exponentially in three dimensions rather than in the linear, template-doubling manner of PCR. Because every new loop can serve as another template, the copy number expands so explosively that fluorescence often reaches a detectable threshold within 8-12 min and plateaus by around 20 min, eliminating the slope information at the beginning and ending phases of the reaction that shows in qPCR, making qLAMP amplification curves even more identical among targets.

This kinetic homogeneity carries practical implications. On the positive side, diagnostic LAMP assays are remarkably rapid. However, that same rapidness limits the amount of discriminative information available in the AC signal. Whereas DDM methods such as ACA can differentiate multiple pathogens in a single fluorescent channel for PCR, the flatter feature characteristics of LAMP curves make target classification considerably harder. Therefore, researchers tend to rely on endpoint strategies to identify the final amplification outputs. These strategies include the use of molecular beacons [180], high-fidelity DNA polymerase-mediated probes [181], labelled primer sets [182], or post-amplification melt analysis [24], to regain specificity. However, unlike PCR, in which probes/beacons can be placed almost anywhere within a longer amplicon, LAMP’s requirement for four to six primers imposes constraints on beacon binding sites, and its need for rapid strand displacement results in limited amplicon sizes. These restrictions on both probe location and amplicon length limit the design of

beacons/probes and achievable separate melting peaks, making higher-order multiplexing challenging.

Following the idea in Chapter 3, a methodology for selecting or modifying AC shapes of qLAMP *in silico* to equip the ACA with easier, distinct targets is required. It is not practical to apply Smart-Plexer strategies directly on existing qLAMP assays, as these unmodified assays will show similar patterns in AC and result in small inter-target distances, which cannot be used by Smart-Plexer. Therefore, this chapter elaborates on modulating qLAMP AC by changing the relative concentration of the primer set. As demonstrated in Figure 6.1(a), unlike shown in qPCR [18], [47], [90], diluted primer concentration does not lead to linear changes of FFI with a clear pattern, probably due to the complex chemical competitions in LAMP, which results in less consumption of primers when the reaction saturates, making the prediction of FFI with primer concentration unreliable.

However, as depicted in Figure 6.1(b), preliminary experiments have indicated robust relationships between increased primer concentration and shortened TTP, which are consistent across a large range of target template concentrations. This inspires the new concept of TTP modulation: with dedicatedly designed primer concentrations, different targets can have non-overlapping ranges of TTP within their Limit of Detection (LOD), based on which a straightforward target identification can be proposed. In an ideal example, Target A with a high primer concentration and fast reactions will show a substantially small TTP at its LOD, which is still smaller than the TTP at Target B's Upper LOD (ULOD), with Target B using a low primer concentration and slower reactions. The essential step of TTP modulation for multiplexing is therefore the selection of primer concentrations for targets, which is not trivial. Unlike the candidate assay selection in Smart-Plexer, the primer concentration is a continuous value, resulting in an infinite number of possible combinations, indicating that selection can only be carried out *in silico*. Furthermore, with an increasing target number, it is impractical to find TTP regions that have no overlap with each other, as chemically, all the reactions can only happen within a certain range of efficiencies. In overlapping regions, DDM methods can be employed to distinguish targets as a supplement to the TTP-based identification. There is a demand for a computation-based design tool to guide and realise this process.

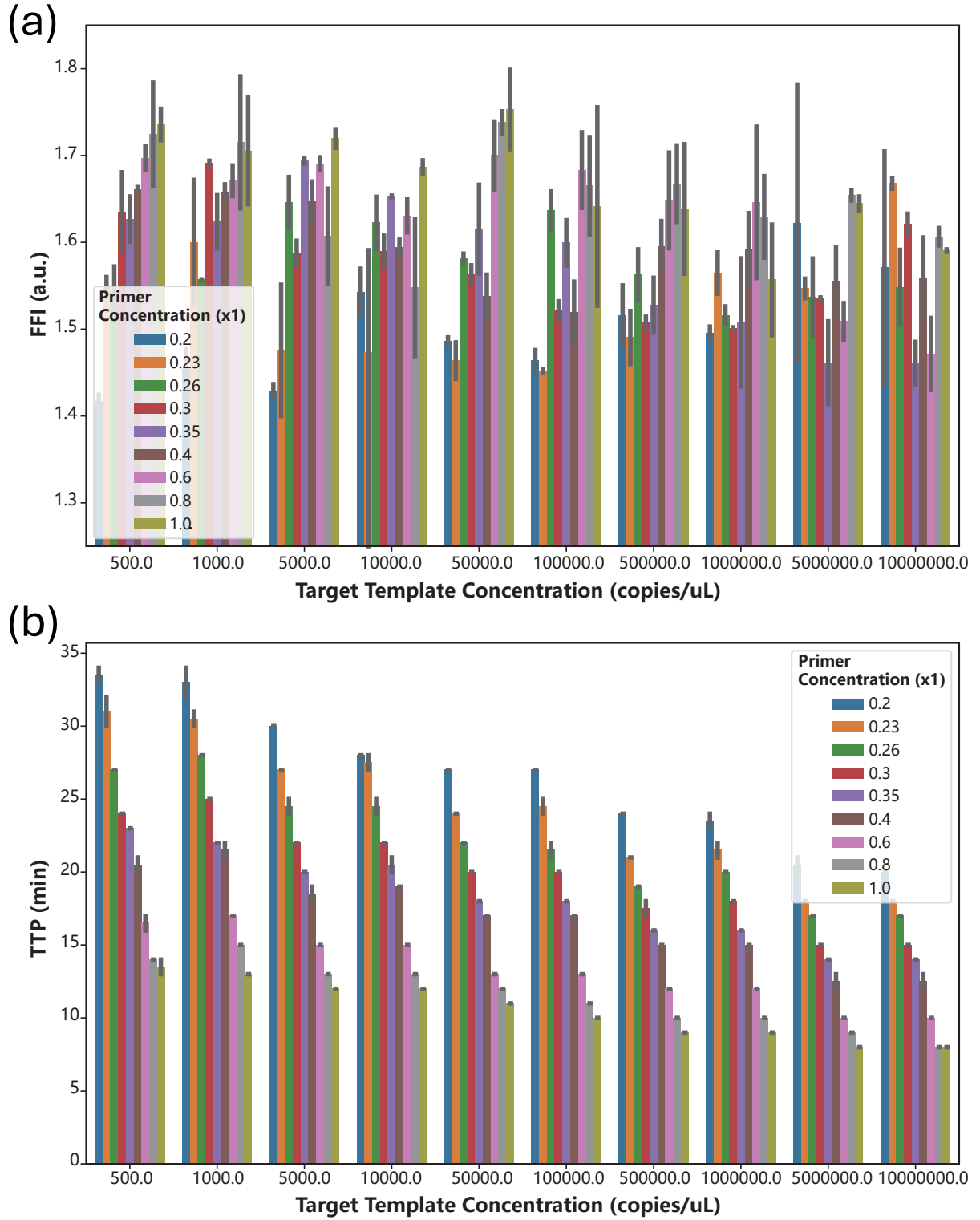


Figure 6.1: Relationships between primer concentration and (a) FFI, (b) TTP across target template concentrations. Error bars indicate the minimum and maximum values.

In this Chapter, a computer-aided assay design framework for qLAMP is proposed. As shown in Figure 6.2, this workflow begins by building a mathematical model to predict the TTP of each target, given the initial target template concentration t and the relative primer set

concentration p . To achieve this, we proposed a two-term power model with six parameters, fitted by simplex data collected with a variety of primer and target concentrations. After generating the target-wise TTP models, we ran global optimisations of primer concentrations according to the Range Overlapping Strategy. Together with post-optimisation criteria, the minimised ranges of overlapping are mathematically assured. The selected assay with optimal primer concentrations was then empirically tested, and ACs with TTP falling into non-overlapping regions were identified directly. Those with TTP falling into overlapping ranges are classified with a two-category ACA, as the proposed optimisation ensured that at most two targets were shown in any TTP region. In this way, the framework divides the complex multi-target ACA problem into numerous simple binary classifications, aiming to improve the overall accuracy of target identification. Results demonstrate the precise fitting and prediction of TTP among multiple targets. Preliminary experiments were carried out, and the results and discussions are provided in 6.4.

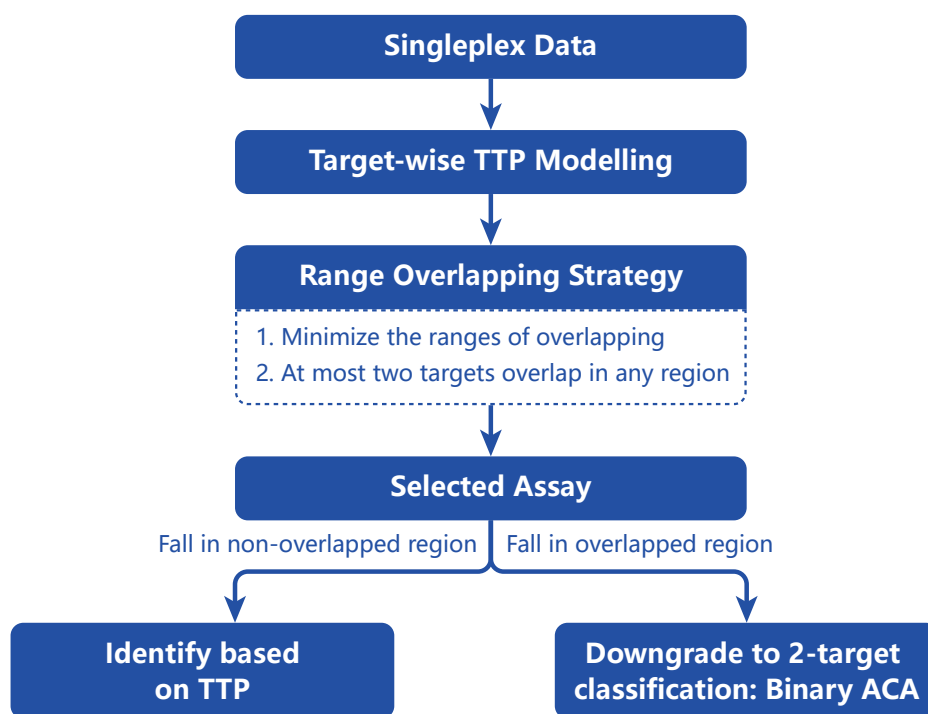


Figure 6.2: Assay design workflow for the proposed TTP modulation. Singleplex data are fitted with a target-wise TTP model against initial template and primer concentration, for in-silico prediction of TTP. A range overlapping strategy is applied to find the optimal assay with minimal ranges of TTP overlapping, ensuring at most two targets overlap in any region. The selected assay is then empirically tested, and ACs with TTP falling into non-overlapping regions are identified directly, whereas those with TTP falling into overlapping ranges were classified with a binary ACA.

6.3 Methodology

6.3.1 Experimental Setups

6.3.1.1 LAMP Reaction Conditions

Real-time LAMP reactions consisted of 10 μL final reaction volume including: 1.00 μL of 10x custom isothermal buffer, 0.50 μL of Mg SO₄ (100 mM stock), 0.56 μL of dNTPs (25 mM stock), 0.30 μL of BSA (40 mg/mL), 1.00 μL of 10x LAMP primer mix, 0.10 μL of NaOH (0.5 M stock), 0.04 μL of Bst 2.0 DNA Polymerase (120 kU/ μL stock), 0.25 μL of Syto9 (20 μM stock), 1.00 μL of sample and enough nuclease free water to have a final volume of 10 μL . Amplification reaction was performed at 63°C for 45 cycles of 60s duration reading at the end of each cycle. LAMP protocol has been adapted from [29].

6.3.1.2 Assays Used in This Chapter

Five species of *Plasmodium* were selected as the target pathogens to detect. Assays of *Plasmodium malariae* (Pm), *Plasmodium ovale wallikeri* (Pow), and *Plasmodium vivax* (Pv) are from [183], assays of *Plasmodium knowlesi* (Pk) from [184], and assays of *Plasmodium falciparum* (Pf) from [185].

To build the mathematical models of TTP, singleplex experiments were conducted for each target under a grid of combinations of primer p and target concentrations t : p in 0.2, 0.23, 0.26, 0.3, 0.35, 0.4, 0.6, 0.8, 1.0 of initial primer concentration ($\times 1$), t in $5 \times 10^2, 10^3, 5 \times 10^3, 10^4, 5 \times 10^4, 10^5, 5 \times 10^5, 10^6, 5 \times 10^6, 10^7$ copies/ μL , in triplicate. p was not evenly selected as TTP tends to show more significant changes in low p regions due to the reduced reaction efficiency, and we wanted to build a precise model with more fine-grained observations. Ranges of t cover the LOD designed for the targets.

After picking the optimal assay combination using the proposed framework, empirical experiments were carried out in triplicate for three of the targets (Pk, Pow, Pv) in both singleplex and multiplex with various target concentrations, to validate the consistency between simulation-based prediction and empirical singleplex and multiplex outputs.

6.3.2 TTP Modelling

We model the TTP vs p and t in a two-term formula,

$$TTP(p, t) = TTP_{baseline}(p) + TTP_{target}(p, t), \quad (6.1)$$

in which $TTP_{baseline}$ is the baseline shift of TTP under a given primer concentration, and TTP_{target} stands for the target concentration-related TTP changes.

As demonstrated in Figure 6.1(b), TTP exhibits a monotonically decreasing trend with the long-tailed ending. To model this process, we tested several non-linear functions, including exponential, logistic, and sigmoidal models, and chose the power function as the final selection based on preliminary results. $TTP_{baseline}$ can then be represented as

$$TTP_{baseline} = ap^b + c, \quad (6.2)$$

where $a > 0$ and $b < 0$.

Like the Ct value in qPCR, the TTP in qLAMP is linearly correlated with the logarithmic value of the initial target template concentration, which can be represented in standard curves [186]. However, the slope of the standard curve can be affected by the primer concentration, due to the shifted reaction efficiency. The lower the primer concentration is, the smaller the negative slope value will become. To model this relationship between slope and p , we applied another power function:

$$Slope(p) = c_1p^{c_2} + c_3, \quad (6.3)$$

with $c_1, c_2 < 0$.

Therefore, the representation of target concentration-related TTP changes is given as

$$TTP_{target}(p, t) = Slope(p) \times \log_{10} t + c' = (c_1p^{c_2} + c_3) \log_{10} t + c'. \quad (6.4)$$

Together with baseline TTP, the joint model is written as

$$TTP(p, t) = ap^b + (c_1p^{c_2} + c_3) \log_{10} t + c_4. \quad (6.5)$$

Note that we merged c and c' as c_4 , the combined constant bias.

For each target, to fit this non-linear joint model with six parameters, we first applied Non-linear Least-Squares (LS) Optimisation with the Trust Region Reflective method to $TTP_{baseline}$ for each template concentration, collecting vectors of fitted a and b as $\mathbf{A}$ and $\mathbf{B}$. The standard curve slope was also fitted with the same algorithm, generating the estimated $\hat{\mathbf{C}} = [\hat{c}_1, \hat{c}_2, \hat{c}_3]$. The optimisation problem is then defined as

$$\begin{aligned}
& \min_{\boldsymbol{\theta}} \text{LS}(TTP) \\
& \boldsymbol{\theta} = [a, b, c_1, c_2, c_3, c_4] \\
& \boldsymbol{\theta}_0 = [\bar{A}, \bar{B}, \hat{C}, 10] \\
& s. t. \quad \mathbf{L} < \boldsymbol{\theta} < \mathbf{H}
\end{aligned} \tag{6.6}$$

where

$$\mathbf{L} = \begin{bmatrix} \max(0, \bar{A} - 5 \times R(\mathbf{A})) \\ \bar{B} - 5 \times R(\mathbf{B}) \\ \hat{C}^T - 5 \\ -20 \end{bmatrix}^T \tag{6.7}$$

and

$$\mathbf{H} = \begin{bmatrix} \bar{A} + 5 \times R(\mathbf{A}) \\ \min(0, \bar{B} + 5 \times R(\mathbf{B})) \\ \hat{C}^T + 5 \\ 50 \end{bmatrix}^T \tag{6.8}$$

in which $R(\mathbf{x}) = \max(\mathbf{x}) - \min(\mathbf{x})$.

The upper and lower bounds were set according to the means and ranges of the parameters estimated in the first step. Although the target function is non-convex, calculating the initial points and feasible regions using the results from conditional optimisations will reduce the fitting difficulty and accelerate the convergence.

6.3.3 Range Overlapping Strategy

As mentioned in 6.2, once the TTP is modelled, a computational strategy is required for selecting the optimal combinations. Figure 6.3(a) illustrates a typical three-plex design scenario, where the standard curves corresponding to the highest and lowest primer concentrations, along with their enclosed areas, are plotted. Selecting a multiplex assay essentially involves choosing standard curves within the enclosed areas for each target. The primary concern is to avoid the TTP overlap among targets as much as possible. An example of possible selection is shown in Figure 6.3(b), where the highest primer concentration for Target A and the lowest for Target B and C are picked, with their corresponding boxplots of distribution depicted. While for Target A, no overlap is shown and the target can be identified straightforwardly, Target B and Target

C suffer from around 25% and 50% of overlap with each other, respectively, stressing the following steps of ACA classification.

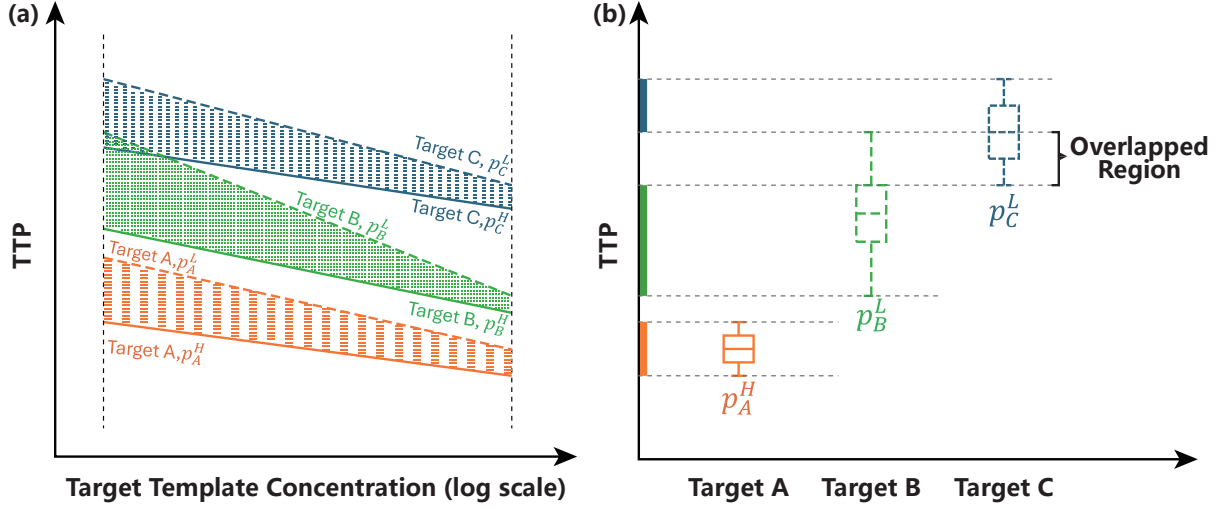


Figure 6.3: Demonstration of Range Overlapping Strategy. (a) Standard curve ranges of three targets within their LODs. (b) TTP range distributions and overlapped regions.

To fully leverage the proposed TTP models in automating the multiplex design, this chapter proposes the Range Overlapping Strategy. There are two criteria for an optimal assay:

- (1) It must maximise the proportions of non-overlapping TTP regions for all the targets.
- (2) It must ensure that at most two targets overlap in any TTP range.

Considering a case of N targets multiplexing, for each Target i , there is a TTP range as

$$R_i = [TTP_i(p_i, t_H), TTP_i(p_i, t_L)], \quad (6.9)$$

in which $TTP_i(p, t)$ is the fitted TTP model for Target i , p_i is the primer concentration to be selected, t_H and t_L are the upper and lower bounds of LOD. Please note that, for simplicity, we use a consistent LOD for different targets in the following derivation, which may be subject to adaptation in real-world applications.

We then define the combined TTP region of all Targets other than Target i as

$$U_i = \bigcup_{j \neq i} R_j, \quad (6.10)$$

and the length of a given range $R = [m, n]$ as its Lebesgue Measure

$$\lambda(R) = |R| = n - m. \quad (6.11)$$

In this way, the percentage of the non-overlapping region within Target i 's total TTP range can be represented as

$$NOLP_i = \frac{\lambda(R_i \setminus U_i)}{\lambda(R_i)}. \quad (6.12)$$

The optimisation problem of maximising the $NOLP$ for all the targets is then given as

$$\begin{aligned} \operatorname{argmax}_{p_1, p_2 \dots p_N} \quad & \min_{i=1, \dots, N} \{NOLP_i\} + \alpha \operatorname{mean}_{i=1, \dots, N} \{NOLP_i\} \\ \text{s. t.} \quad & p_i \in [p_i^L, p_i^H], i = 1, \dots, N \\ & \alpha > 0 \end{aligned} \quad (6.13)$$

The first term of the objective function emphasises the importance of enlarging the minimum overlapping percentage, as the bottleneck of classification is ultimately constrained by the weakest element, much like how a barrel's capacity is limited by its shortest plank. Additionally, the second term also considers the overall performance across all targets, thereby preventing the solution from falling into a local minimum of the objective function. In this chapter, α is fixed as 0.1 to achieve a trade-off.

Since the objective function is non-analytic and non-convex, Particle Swarm Optimisation (PSO) [187] was applied to find the global optimal solution in the feasible region, using 1000 particles, a cognitive parameter of 0.4, a social parameter of 0.2, and an inertia parameter of 0.6, over 500 iterations. The PSO algorithm was run ten times with randomly generated initial parameters to find the optimal solution.

A post-optimisation check was then adopted to examine the second criterion, ensuring a maximum of two targets overlap in any TTP region.

6.3.4 Empirical Tests and Target Identification

Empirical multiplex tests were conducted on the selected combination of primer concentrations. Ideally, the ranges of TTP within LOD should match the simulated ones for all the targets. Reactions with TTP falling into the non-overlapping regions shall be identified directly, whereas those in the overlapping areas will be input into the standard ACA algorithm [22].

6.4 Results and Discussion

6.4.1 TTP Modelling Results

Figure 6.4(a)-(c) demonstrates the fitting results of Target ‘Pm’. As shown in Figure 6.4(a), TTP baseline models present excellent fitting performance across all the target concentrations, indicating the model’s robustness under different reaction efficiencies. Quantitative fitting errors of TTP vs primer concentration are provided in Table 6.1, with all the targets presenting average Root Mean Squared Error (RMSE) smaller than 0.8 min. It is noticed that fitting errors tend to be larger in lower target concentrations, and for Pow, no positive reactions are observed below 5×10^3 copies/ μ L, indicating that reactions become less robust and introduce more randomness in low efficiencies. Nevertheless, the largest RMSE is still under 1.6 minutes. These precise TTP predictions confirm the feasibility of using power functions in revealing the non-linear relationship between efficiency drop and reduced primer concentrations.

Figure 6.4(b) further illustrates how well the standard curve slope is simulated with the $TTP_{target}(p, t)$, supported by the small fitting errors of slopes across all targets in Table 6.2. The 3-D mapping of TTP vs p and t for Target Pm is presented in Figure 6.4(c). This twisted fitting surface indicates the complex nature of the primer concentration’s influence on the reaction speed, emphasising the significance of using the proposed two-term model. The surface matches the observations precisely, endorsed also by the negligible fitting errors shown in Table 6.2.

When plotting a series of predicted standard curves together, they form a Sector-like quadrilateral, as depicted in Figure 6.4(d). As these standard curves correlate well with experimental results (data points in the figure), the combined TTP model can also be used in predicting any standard curve within the quadrilateral.

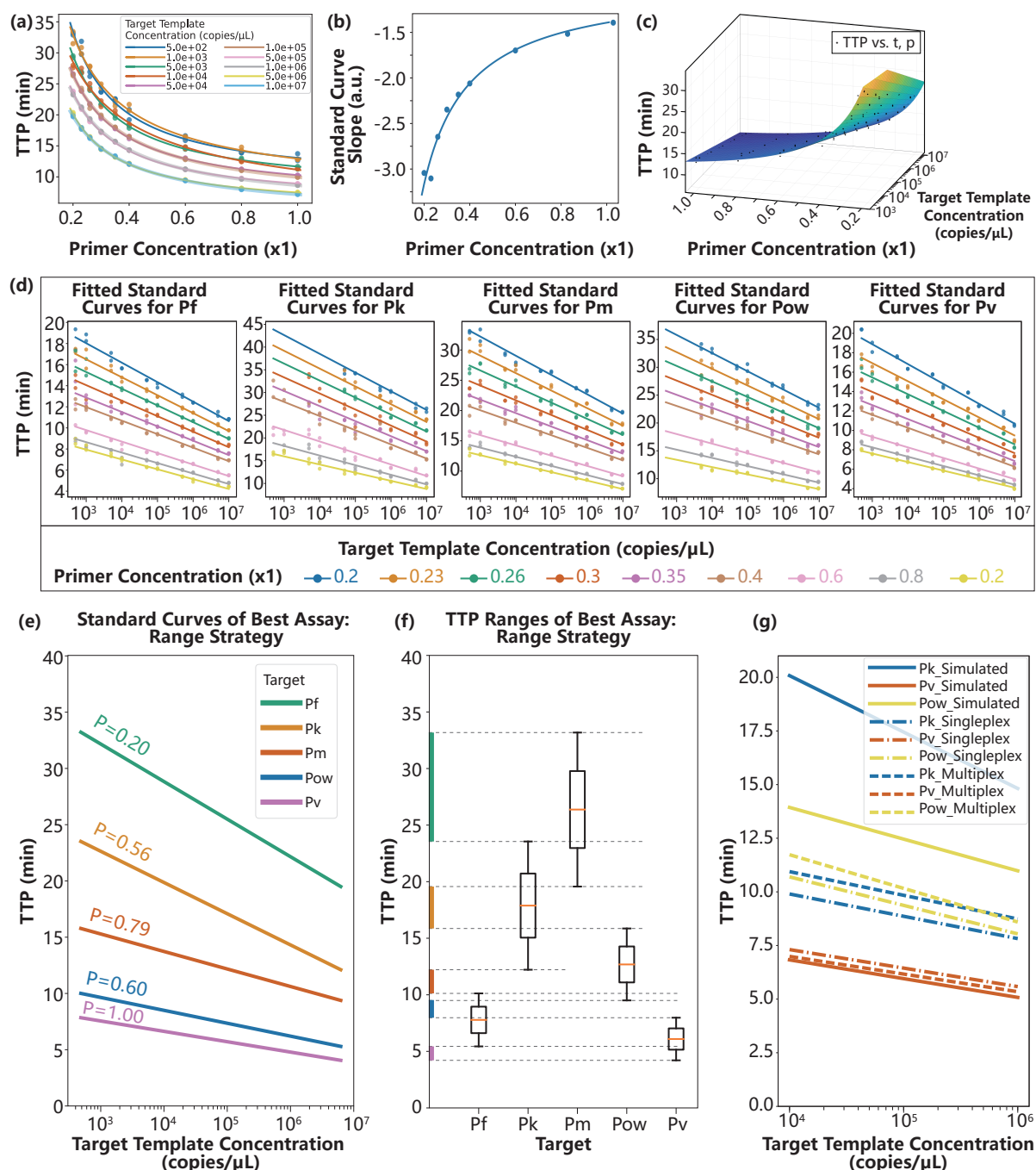


Figure 6.4: Results of the proposed TTP modulation. An example fitting result of target Pm: (a) TTP vs primer concentration across ten target template concentrations; (b) Standard curve slope vs primer concentration; (c) TTP vs primer and target concentrations. (d) Fitted standard curves for five targets, under different primer concentrations. (e) Standard curves of the selected optimal assays. (f) Boxplots of TTP in optimal assays. (g) Simulated, empirically tested singleplex, and empirically tested multiplex standard curves.

Table 6.1: Fitting errors of TTP vs primer concentration.

RMSE (min)		Target Template Concentration (copies/ μ L)										Mean
		5×10^2	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6	5×10^6	10^7	
Target Name	Pf	0.9656	0.6974	0.2495	0.2909	0.1646	0.1055	0.0863	0.0697	0.0867	0.0610	0.2777
	Pk	1.1519	0.7188	1.1264	1.1758	0.8368	0.8604	0.9377	0.2934	0.3847	0.3060	0.7792
	Pm	0.7299	0.6123	0.2383	0.5405	0.1075	0.2307	0.1349	0.0826	0.1181	0.0957	0.2891
	Pow	/	/	0.8216	0.6224	0.4715	0.4167	0.2198	0.2581	0.2175	0.2408	0.4085
	Pv	0.4316	0.3003	0.1477	0.1149	0.1173	0.0906	0.0868	0.0915	0.1091	0.1118	0.1602

Table 6.2: RMSE of slope and combined fitting.

	Target Name					Mean
	Pf	Pk	Pm	Pow	Pv	
RMSE Slope Fitting (a.u.)	0.1070	0.2517	0.0907	0.1052	0.0345	0.1178
RMSE Combined Fitting (min)	0.4714	1.1183	0.5513	0.5768	0.3425	0.6121

6.4.2 Selected LAMP Multiplex Assay

Standard curves and TTP distributions of the optimal assay are provided in Figure 6.4(e). Standard curves that are relatively flat (i.e., those with a small absolute slope and a narrow span between their highest and lowest TTP) are positioned close to one another by the algorithm. In contrast, curves that drop more sharply (with a larger negative slope) and cover a wider TTP range are placed farther apart. This trend of the algorithm enables a relatively large non-overlapping percentage for all the targets, emphasised by the box plots in Figure 6.4(f), in which all the targets are assured at least 35% of TTP regions that can be directly used for identification. This strategy of maximising the minimum overlap helps maintain a consistent probability of misidentification among targets, thereby benefiting the overall accuracy.

6.4.3 Empirical Results and Discussions

Pk, Pow, and Pv were selected for the preliminary empirical experiment, testing in both singleplex and multiplex. The fitted and empirical standard curves of these targets are shown in Figure 6.4(g). Pv demonstrates an ideal situation, in which the simulated TTP correlates well with singleplex and multiplex results across all the target concentrations, with errors smaller than 0.5 min, enabling precise TTP-based discrimination. For Pk and Pow, simulated reactions

are much slower than the empirically tested ones. Considering the fact that the simulation fits perfectly with previous singleplex data, this inconsistency may arise from experimental factors such as human errors or stock issues other than fitting failure. Further efforts will focus on smoothing the experimental procedures and generating high-quality amplification data for further validation of the method. Nevertheless, empirical singleplex and multiplex data are showing a TTP gap of less than 1 min, validating the assumption of TTP consistency when transferring from singleplex to multiplex environments. Therefore, with dedicatedly conducted experiments, it is promising that the proposed method will provide highly accurate TTP prediction and better target identification.

It is worth mentioning that this strategy may not be feasible with real-time PCR, because the cycle-based chemistry produces amplification curves within a broad but overlapping C_t window due to its relatively low reaction speed compared with LAMP, regardless of initial primer concentration. As a result, real-time PCR cannot segregate targets into distinct detection bands based on C_t alone. In contrast, LAMP's isothermal, continuous amplification kinetics create discrete time-to-positive ranges for different targets, enabling clear separation and identification without overlap.

6.5 Conclusion

In conclusion, this chapter presents a novel computational framework designed to improve multiplex detection in qLAMP assays. By systematically adjusting the relative concentrations of LAMP primers, the method enables modulation of amplification curve TTP, without relying on changes in fluorescence saturation intensity. This TTP modulation facilitates the temporal separation of targets into distinct, non-overlapping detection windows, offering a strategic approach for multiplexing. A two-term power model was developed to characterise the relationship among primer concentration, template concentration, and TTP, enabling global optimisation of primer concentrations to reduce TTP overlap. In instances where temporal separation is insufficient, a binary ACA is employed to resolve ambiguities, thereby converting a complex multi-class classification problem into a series of manageable binary decisions. The empirical results underscore the robustness and scalability of this hybrid strategy, demonstrating its practical potential to streamline and automate qLAMP assay design in multiplex molecular diagnostics. Future work will aim to refine the experimental protocols and generate high-quality amplification data to support further validation and optimisation of the proposed methodology.

Chapter 7 Conclusion and Future Perspective

7.1 Thesis Conclusion

This thesis pushes the practical and theoretical boundaries of multiplex molecular diagnostics by fusing assay chemistry with advanced computational intelligence. Across five sequential studies, it moved from the fundamental challenge of distinguishing valid from spurious amplification events, through computer-aided assay development and deep domain adaptation enhanced ACA, to fully automated, point-of-care implementations and pioneer research of target identification in isothermal chemistries. Although each chapter addresses a distinct technical hurdle, together they form an integrated research arc that advances the accuracy, scalability and clinical readiness of nucleic-acid detection platforms.

Chapter 2 lays the analytic foundation with the **Adaptive Mapping Filter (AMF)**, a kinetic-aware outlier-detection framework for qdPCR. By profiling entire amplification curves rather than relying solely on endpoint fluorescence, AMF discerned non-specific or low-efficiency reactions that confound conventional thresholding. When deployed for rapid carbapenemase gene surveillance, the filter increased sample-level sensitivity while cutting false-positive and false-negative rates. Beyond improving an individual test, AMF demonstrated that harnessing reaction kinetics can transform raw fluorescence readouts into meaningful diagnostic signals. This principle of kinetic feature mining inspires subsequent contributions.

Building directly on that insight, Chapter 3 introduced **Smart-Plexer 2.0**, a computational development environment for multiplex real-time PCR. The platform expands the feature space used for primer set selection, adding higher-order kinetic descriptors and a clustering-based distance metric to quantify cross-target separability. Compared with its predecessor, Smart-Plexer 2.0 delivered tighter performance variance across selected optimal assays and retained high accuracy under laboratory noise. Crucially, the study showed that computational optimisation can compensate for complex amplification dynamics, enabling larger target panels without additional fluorescent channels. In addition, Smart-Plexer translates the theoretical gains of AMF into a practical assay-development pipeline.

While Chapter 2 and Chapter 3 focus on assay optimisation within a single data domain, Chapter 4 tackles the cross-domain generalisation problem. The proposed **Transformer-Conditional Domain Adversarial Network (T-CDAN)** transfers classifiers trained on abundant in-silico DNA curves to scarce clinical dPCR datasets. Its transformer encoder

captures long-term temporal patterns, whereas adversarial training minimises distributional discrepancy. Tested on 198 antimicrobial-resistant bacterial isolates, T-CDAN achieved 93.1 % curve-level and 97.0 % sample-level accuracy, significantly outperforming classical machine learning baselines. The ability to leverage synthetic data not only reduces the cost and biosafety burden of generating lab datasets, but also paves the way for adaptive diagnostics that remain current as pathogen genomes evolve.

Chapter 5 further leverages algorithmic advances with an end-to-end **AI-driven cLAMP workflow** suitable for resource-limited settings. An unsupervised hybrid segmentation-and-cleaning algorithm flow analysed tube-strip photographs captured with portable devices, rejecting invalid assays with > 96 % accuracy and identifying colour outcomes with above 99 % accuracy in real clinical samples. This work demonstrates that pairing low-cost chemistry with advanced image processing and AI can decentralise high-complexity multiplex diagnostics, representing an essential attribute for outbreak containment and routine surveillance in low-infrastructure environments.

Recognising that colorimetric readouts cannot yet discriminate multiple targets in a single reaction, Chapter 6 returns to reaction engineering with a **TTP modulation framework for qLAMP**. By systematically adjusting primer concentrations, we introduced predictable temporal offsets between target reactions. Built on a multi-variable non-linear TTP model, the proposed Range Overlapping Strategy selected primer concentrations that maximise inter-target TTP separation without sacrificing amplification efficiency. When residual overlap occurred, an auxiliary binary ACA resolves ambiguous cases. Ideally, the resulting hybrid system will maintain high classification accuracy while preserving the rapid reaction speed and isothermal simplicity of LAMP, illustrating a new route to high-order single-channel single-well multiplexing in qLAMP.

Taken together, these chapters constitute a coherent strategy for information-centred data-driven molecular diagnostics. Instead of accepting assay physics as a fixed constraint, this thesis treat amplification curves as rich data sources for signal processing, machine learning and optimisation. Unsupervised outlier removal (Chapter 2) improves base-level data quality; feature-space expansion and clustering (Chapter 3) guide assay selection; representation learning and domain adversarial training (Chapter 4) generalise classifiers across settings; computer-vision pipelines (Chapter 5) bring automation to POC formats; and non-linear modelling plus non-convex global optimisation (Chapter 6) extend multiplex capacity without additional hardware. Each level of them reinforces the others, contributing to diagnostic workflows that are accurate, scalable and cost-effective.

In conclusion, this thesis advances multiplex molecular diagnosis from both a biochemical and an informatics perspective, demonstrating that thoughtful integration of adaptive filtering, machine-driven design, domain-robust learning, and cloud-scale automation can substantially improve diagnostic precision and accessibility. By showing how each layer of the analytical stack can be optimised, the work provides a blueprint for next-generation assays that are not only faster and more sensitive but also smarter—capable of learning from every output they generate.

7.2 Future Perspective

The ultimate goal of this thesis is to translate the proposed methodologies into practical tools that advance global health. Nevertheless, several domains still require sustained exploration and refinement:

Data perspective: Although variance was deliberately introduced by modulating target concentrations, reagent compositions, and operational parameters, the real circumstances of conducting chemical reactions will always shift the sensitive optical outputs of amplifications. Further efforts will focus on the standardisation of operation and reaction environments for reproducible assay performance, and the development of ACA strategies resilient to experimental variability. In addition, dedicatedly designed clinical studies are needed to accumulate larger datasets, thereby supporting comprehensive validation and iterative enhancement of the algorithms.

Particularly, in human pathogen diagnostics, switching the clinical sample sources, such as blood, saliva, stool, urine, or sputum, can shift real-time PCR amplification curves (e.g., delayed C_t , reduced slopes, lower FFI) because sample components alter polymerase activity and fluorescence detection [103]. Blood-derived specimens often delay or flatten curves because haemoglobin and IgG inhibit Taq Polymerase and can quench fluorescent dyes [188]. Saliva consistently produces a positive C_t shift with large person-to-person variability, reflecting mucins, enzymes, and other inhibitors in the sample [189]. Faecal samples frequently carry complex polysaccharides that block amplification, yielding low-amplitude or delayed curves [190]. Urine introduces urea and other solutes that reduce amplification efficiency, shifting kinetics unless extraction and buffer conditions are optimised [191]. Viscous sputum contains undefined inhibitors, so C_t values may not track pathogen load, causing inconsistent curve shapes [192]. High background human genomic DNA or co-amplified non-target

sequences can also distort curves by consuming primers/polymerase and prolonging C_t [193]. Sample-induced shifts in pH or ionic strength away from the Taq optimum ($\sim$ pH 8-9) slow extension and change curve slopes [194]. Consequently, apparent curve differences may reflect matrix chemistry rather than true pathogen kinetics, underscoring the need for internal inhibition controls and matrix-specific optimisation strategies [103].

Methodology perspective: Additional effort is needed to extend the proposed techniques to broader contexts, potentially with context-specific adjustments. For example, AMF could be employed to clean amplification curves from conventional qPCR and qLAMP assays; T-CDAN may prove effective for other domain gaps, such as transferring from digital to bulk platforms or adapting across different instruments; and the automated cLAMP pipeline might be applicable to alternative chemistries such as RCA. Lastly, building on promising preliminary results, the ongoing study of TTP modulation is expected to open new avenues for data-driven multiplexing in LAMP.

Co-amplification issue: In clinical practice, it is common to encounter samples containing multiple pathogens. However, this thesis does not address the scenario of co-amplification in ACA, where more than one target is present and is amplified within a single reaction well. Overcoming this limitation of the current ACA framework will be a key focus of our future work, following a structured approach: (1) Assay Design to Minimise Single-Well Co-Amplification: in real-world applications, ACA will be integrated with conventional spatial and spectral multiplexing strategies. Clinical knowledge of pathogen pairs or clusters that frequently co-occur in individual samples will be leveraged to guide assay design [195]. Specifically, targets with a high likelihood of co-existence will be assigned to different wells or detection channels, thereby statistically reducing the probability of co-amplification within a single well. (2) Detection of co-amplification using FFI: as demonstrated by Rajagopal et al. 2019 [47], a significant FFI increase in real-time PCR can be spotted when multiple targets are amplified simultaneously, and the value of FFI is ideally decided by the sum of individual targets' FFI in TaqMan chemistry, assuming that all the primers of the amplified targets are consumed at the end of the reaction. With carefully designed primer and probe concentrations, FFI can potentially serve not only as an indicator of co-amplification but also as a quantitative measure for estimating the number of co-amplified targets, and possibly for their identification [18], [90]. (3) Decoupling amplification curves: theoretically, a co-amplified AC can be regarded as the additive result of the individual target-specific ACs. By employing joint

parametric modelling to estimate the parameters representing the combined curve, it may be possible to deconvolute the observed AC into separate target-specific ACs [124]. These reconstructed ACs can then be analysed using pretrained ACA models for target identification. Nonetheless, this additive model may be confounded by competitive chemical interactions and variable reaction efficiencies, potentially leading to distorted decomposed ACs. In such cases, domain adaptation strategies, as discussed in Chapter 4, can be applied to mitigate discrepancies between the pretrained models and real-world decomposed outputs.

7.3 Final Remarks

Recent years have witnessed computer scientists turning to infectious-disease problems, just as experimental biologists have begun embracing large-scale data analytics. The work presented in this thesis is the product of an interdisciplinary effort spanning signal (including image) processing, machine learning, software engineering, analytical chemistry, and clinical microbiology. Given the current situation, where multiplex molecular diagnostics still lack cohesive and data-driven design principles, the chapters herein aim to close that gap. Together, these advances demonstrate how kinetic signal processing and modern artificial intelligence can transform raw amplification outputs into clinically actionable knowledge, without imposing hardware barriers. It is my hope that by making these ideas, algorithms, and data publicly available, researchers and practitioners will adapt, refine, and scale them to confront the most pressing global health challenges that lie ahead. We will always continue preparing for the next inevitable pandemic.

Appendix A Supporting Information for Chapter 2

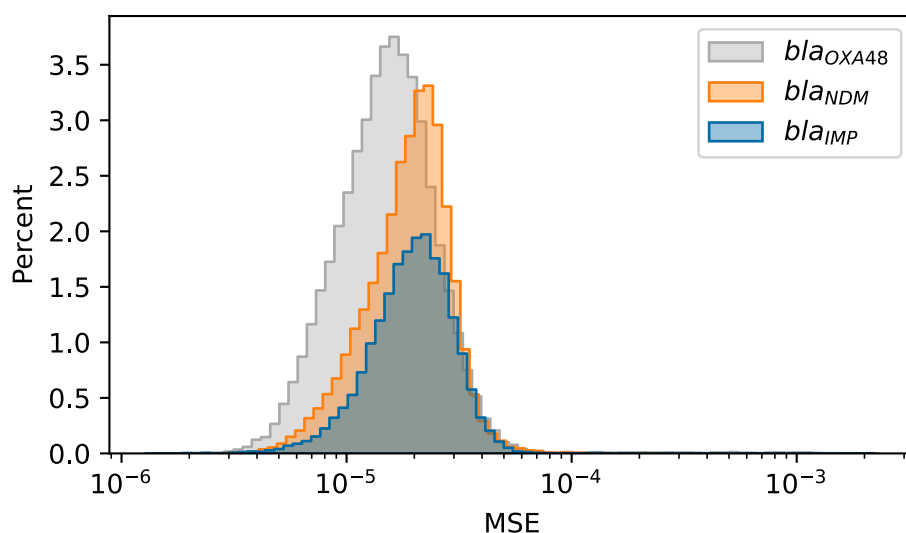


Figure A.1: MSE distribution. MSE is used to monitor the fitting performance and the quality/correctness of fitted parameters. As shown in the figure, the majority of curves have an MSE smaller than 10^{-4} , indicating good fitting considering the fluorescence values ranged between 0 and 1.

Table A.1: Curve-level performance comparison between the original ACA method (45 cycles + KNN) and the proposed 5 parameters + Random Forest method, before applying AMF.

	Precision (%)		Sensitivity (%)		F1-score	
	KNN	RF	KNN	RF	KNN	RF
<i>bla</i> _{NDM}	65.5	80.0	83.7	94.4	0.73	0.87
<i>bla</i> _{IMP}	67.8	75.3	96.0	97.8	0.80	0.85
<i>bla</i> _{OXA-48}	84.4	94.8	52.8	70.6	0.65	0.81
Accuracy (%)					71.7	83.9

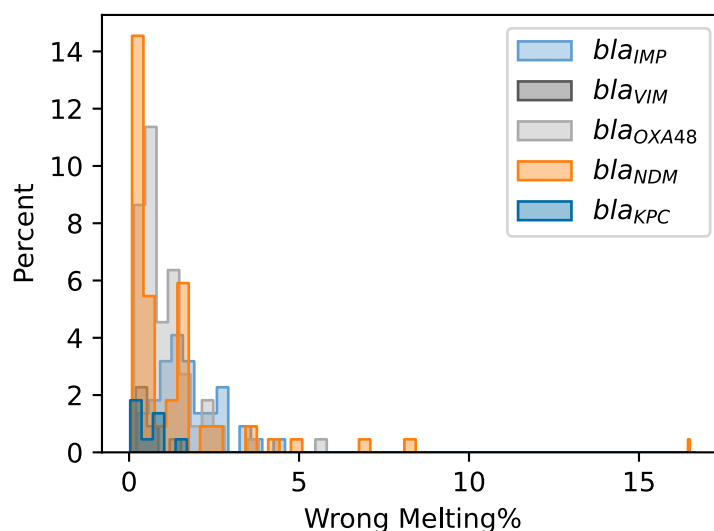


Figure A.2: Outlier distribution in clinical samples. The X-axis shows the percentage of the wrong melting calculated from our algorithm, and the Y-axis shows the outlier percentage.

Section A.1 Assay Design Strategy

Design and in-silico analysis were conducted using GENEious Prime 2020.1.2 (<https://www.geneious.com>). Primer features were analysed through IDT OligoAnalyzer software (<https://eu.idtdna.com/pages/tools/oligoanalyzer>) using the J. SantaLucia thermodynamic table for melting temperature (T_m) evaluation, hairpin, self-dimer, and cross-primer formation (<https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>). The T_m of the amplification product of each gene was determined by Melting Curve Predictions Software (uMELT) package (<https://dna-utah.org/umelt/umelt.html>).

Appendix B Supporting Information for Chapter 3

Section B.1: Materials and Methods

Double-stranded synthetic DNA was used in this chapter to develop and assess the performance of all singleplex assays. In particular, the entire coding sequences of the following strains were used: A/H1N1/09, A/H1N1, A/H3N2, hIAV, hIBV, hRSV, Spn. The following NCBI accession numbers were used as references for the gBlocks synthesis: CY181417, MN055282, NC_007366, NC_002016, NC_002204, NC_001803, NC_003098, respectively. Based on the comprehensive analyses and alignments of each type using the MUSCLE algorithm [196], primers were specifically designed to amplify all sequence variations within each gene belonging to their specific target (inclusivity) and to exclude closely related but not inclusive sequences (exclusivity). Design and in-silico analysis were conducted using Geneious Prime 2022.0.1 [197]. Oligonucleotides and synthetic constructs (length ranging from 747 to 1,775 bp) were purchased from Integrated DNA Technologies Ltd. (IDT) and resuspended in Tris-EDTA buffer to 10ng/ μ L stock solutions (stored at -80°C until further use). The concentrations of all DNA stock solutions were determined using a Qubit 3.0 fluorimeter (Life Technologies).

For real-time amplification experiments, we used the BioMark HD (Fluidigm). The master mix used was the PrimeTime Gene Expression Master Mix from Integrated DNA Technologies (IDT, catalog no. 1055772) supplemented with ROX passive reference dye and pre-mixed following the manufacturer's guidelines. The qdPCR was performed with Fluidigm qdPCR 37k integrated fluidic circuits (IFC) (catalog No. SKU100-6152) and was supplemented with Fluidigm 20X GE loading buffer (PN 85000746). The priming and loading steps of the IFC were followed as the supplier's protocol (Fluidigm document number: 100-6896 Rev 03). Each amplification mix for the qdPCR experiment contained 3 μ L 2X IDT PrimeTime Gene Expression Master Mix (with passive ROX), 0.6 μ L 20X GE, 0.6 μ L 10X Primer mixture, 1.8 μ L DNA templates from synthetic DNA, pre-amplified cDNA, or controls, and brought the final volume to 6 μ L. A total of 4.5 μ L of reaction mix was transferred to each inlet (or panel) of a Fluidigm 37k IFC for the thermal cycling step. Thermal-cycle conditions consisted of a hot start step for 3 min at 95°C, followed by 45 cycles at 95°C for 15 seconds and 60°C for 45 seconds. Real-time data of the amplification events were exported as a text file for each bulk by Fluidigm Digital PCR Analysis software (version 4.1.2).

Table B.1: Primer tables [21]. Adapted under CC BY-NC 4.0 [55].

Primer_ID	Primer Sequence	Strain
9H101	TACCCAAARCTCAGCAAATCCT	A/H1N1/09
9H103	AGACTTTGTTGGTCAGCACTAGT	A/H1N1/09
AH101	AATTGGGTAAATGCAGCGTTGC	A/H1N1
AH103	GTKTTTCTACAATGTAGGACCATG	A/H1N1
AH104	CCCTGGGTAAACATGTTCCATTCTC	A/H1N1
AH301	AGCAACTGTTACCCTTATGATGT	A/H3N2
AH302	TATGATGTGCCGGATTATGCCT	A/H3N2
AH304	CAGTCCAATTGAAGCTTTCATTGT	A/H3N2
AH305	TGAGTGACTCCAGTCCAATTGA	A/H3N2
FLA01	TGCAGTCCTCGCTCACTGGGCACG	hIAV
FLA02	TGCAGTCCTCGCTCACTGGGCACG	hIAV
FLA16	ACCAATCCTGTCACCTCTGACT	hIAV
FLA17	GGCATTTTGGACAAAGCGTCT	hIAV
FLB01	GATCGAATCTGCACTGGRATAAC	hIBV
FLB03	TGCTACTCAAGGGGARGTCAA	hIBV
FLB05	GTGGGTGTTGTTGTCAGTGGTAT	hIBV
FLB07	CCYCTGGTTTCTGTTCTTTGA	hIBV
FLB08	GGCTACRTCCAGATCTGTGCA	hIBV
RSV01	GCAAATATGGAAACATACGTGAACA	hRSV
RSV03	GCACCCATATTGTWAGTGATGCA	hRSV
RSV04	AAAGACGATGAYCCTGCATC	hRSV
RSV06	AGCACTGCACTTCTYGAGTT	hRSV
SPN01	ACGCAATCTAGCAGATGAAGCA	Spn
SPN03	CTCGTGCGTTTTAATTCCAGCT	Spn

Target	gBlocks	Assay_ID	Forward_ID	Probe_ID	Reverse_ID	Comments
9H1	A2009_HA_x_CY181417	9H1_HA_02	9H101	9H104	9H103	
9H1	A2009_HA_x_CY181417	9H1_HA_03	9H101	9H105	9H103	
AH1	AH1N1_HA_x_MN055282	AH1_HA_03	AH101	AH105	AH103	
AH1	AH1N1_HA_x_MN055282	AH1_HA_04	AH101	AH105	AH104	
AH1	AH1N1_HA_x_MN055282	AH1_HA_05	AH101	AH106	AH103	
AH1	AH1N1_HA_x_MN055282	AH1_HA_06	AH101	AH106	AH104	
AH3	AH3N2_HA_x_NC_007366	AH3_HA_03	AH301	AH307	AH304	
AH3	AH3N2_HA_x_NC_007366	AH3_HA_04	AH302	AH307	AH305	
RSV	ReSyV_M1_x_NC_001803	RSV_M1_03	RSV01	RSV07	RSV03	
RSV	ReSyV_M1_x_NC_001803	RSV_M1_04	RSV04	RSV08	RSV06	
SPN	Strpn_lytA_x_NC_003098	SPN_LYT_02	SPN01	SPN04	SPN03	
FLB		FLB_HA_05	FLB01	FLB09	FLB05	
FLB		FLB_HA_06	FLB03	FLB10	FLB05	
FLB		FLB_HA_07	FLB01	FLB11	FLB07	
FLB		FLB_HA_08	FLB03	FLB11	FLB08	NOT USED
FLA	Flu-A_M12_x_NC_002016	FLA_M_01	FLA16	FLA01	FLA17	
FLA	Flu-A_M12_x_NC_002016	FLA_M_02	FLA16	FLA02	FLA17	

	9H1	AH1	AH3	FLA	FLB	RSV	SPN
PM7.078	9H1_HA_03	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_05	RSV_M1_04	SPN_LYT_02
PM7.013	9H1_HA_02	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_05	RSV_M1_03	SPN_LYT_02
PM7.014	9H1_HA_02	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_05	RSV_M1_04	SPN_LYT_02
PM7.077	9H1_HA_03	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_05	RSV_M1_03	SPN_LYT_02
PM7.016	9H1_HA_02	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_07	RSV_M1_04	SPN_LYT_02
PM7.080	9H1_HA_03	AH1_HA_03	AH3_HA_04	FLA_M_02	FLB_HA_07	RSV_M1_04	SPN_LYT_02

Table B.2: Key packages of the development environment.

Name	Version
python	3.9.17
numpy	1.22.4
pandas	1.5.3
matplotlib	3.7.1
seaborn	0.12.2
scipy	1.10.1
scikit-learn	1.2.2

Table B.3: Running time for Smart-Plexer algorithms (34650 reactions).

Name	Time Period (s)
Pre-Processing & Sigmoidal Fitting	148.6
Adaptive Mapping Filtering	4.3
Feature Extraction	4.2
Distance Calculation (S1)	0.8
Distance Calculation (S2)	1
Distance Calculation (S3)	217.3
Ranking	0.1

Table B.4: Running time for ACA algorithms (41128 reactions).

Name	Time Period (s)
Pre-Processing & Sigmoidal Fitting	194.2
Adaptive Mapping Filtering	4.8
Feature Extraction	4
Feature Normalisation	22.1
Classification	1.8

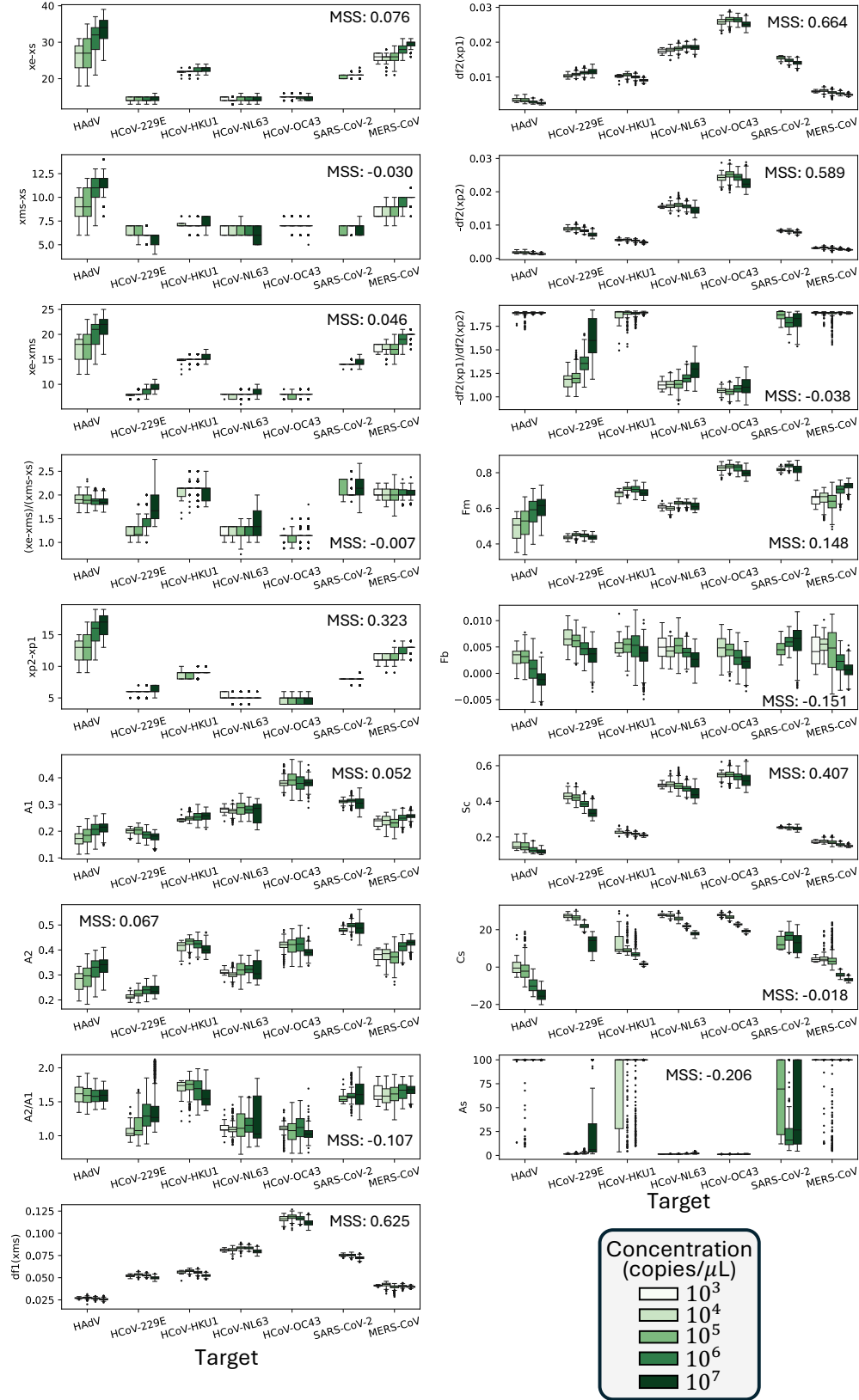


Figure B.1: Feature distributions across concentrations on SP1-7-plex-RTI dataset. Box plots of various target types and concentrations are shown. The X-axis shows different pathogen DNA targets, and distributions of different target concentrations are depicted in shades of colours and grouped in subplots. Mean Silhouette Scores among target clusters are also presented in the upper-right corner, for each feature.

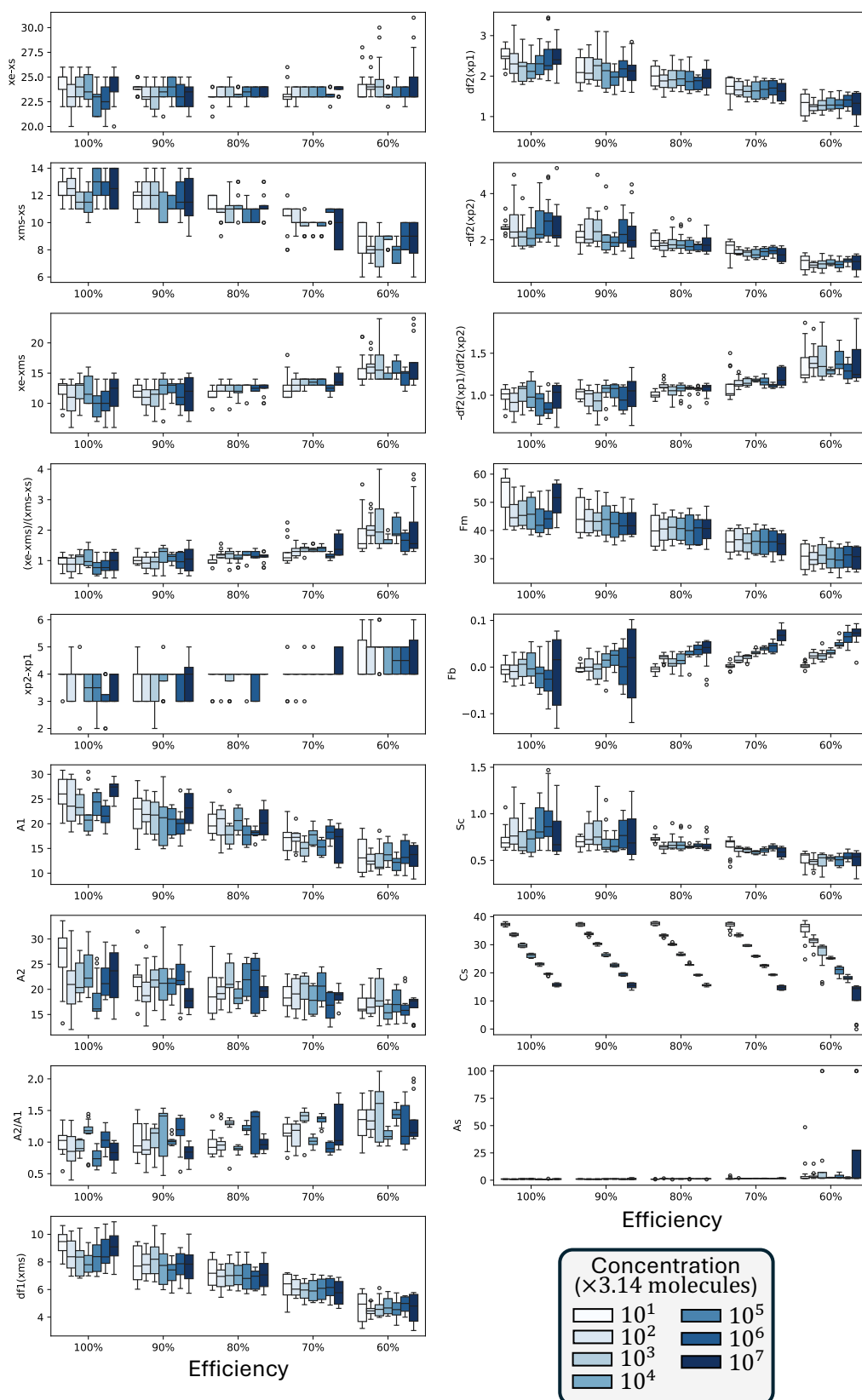


Figure B.2: Feature distribution consistency among concentrations under different reaction efficiencies tested on the Ex-Conc-Eff dataset. Box plots of various target concentrations and reaction efficiencies are shown. The X-axis shows different reaction efficiencies, and distributions of different template concentrations are depicted in shades of colours and grouped in subplots.

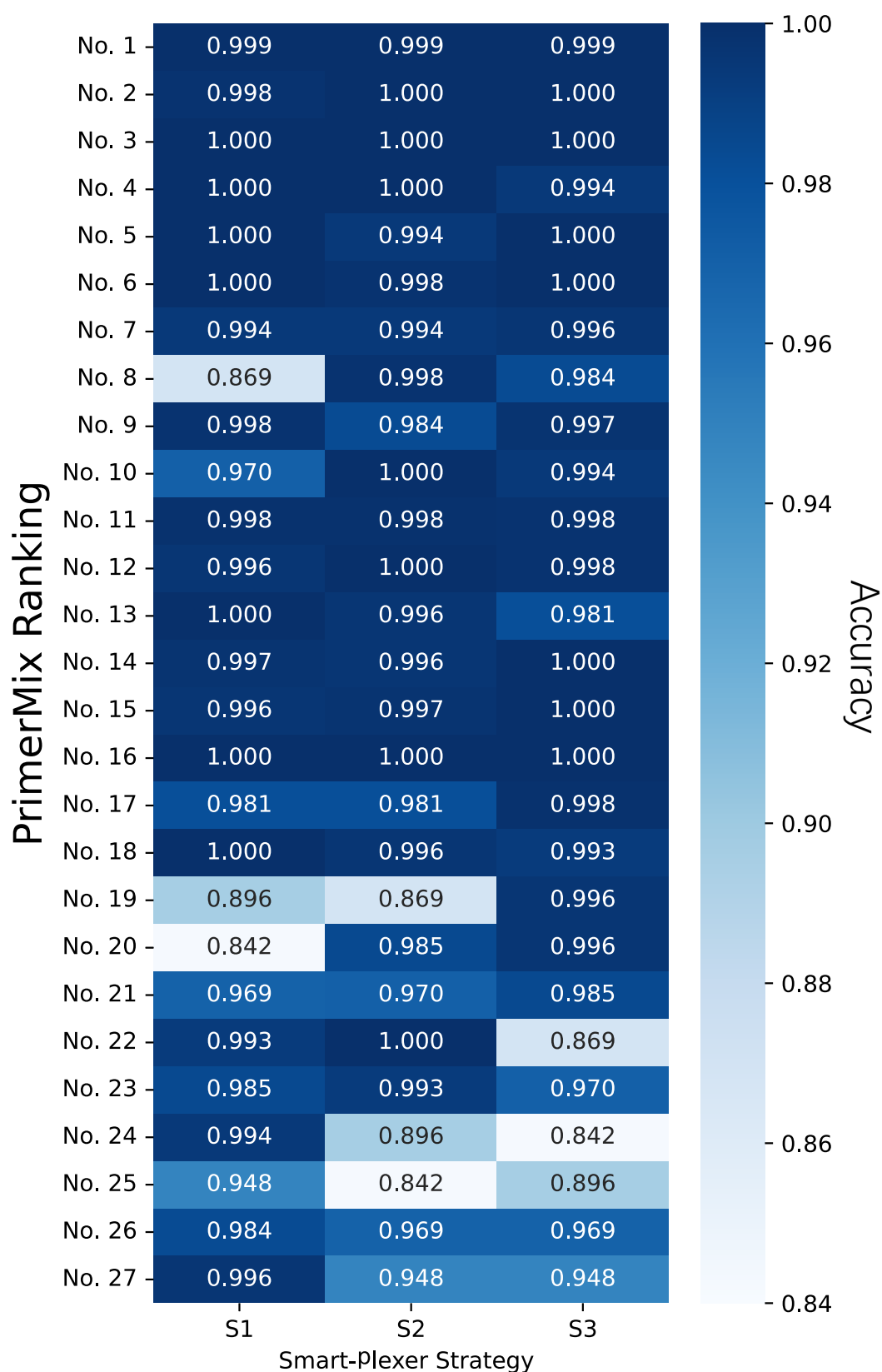


Figure B.3: Ranking-accuracy mapping of SP1-3-plex-RTI dataset. From top to bottom, each column shows the real-world ACA accuracies of the multiplexes ranked from the first to the last, in both numerical values and colours. Ideally, the higher the rank, the better performance and accuracy it will present. S2 and S2 follow this assumption, but S1 shows a complex pattern with highly ranked assays providing unexpectedly worse ACA performance, e.g., No. 8.

Appendix C Supporting Information for Chapter 4

Table C.1: Curve-level results with four benchmark algorithms.

Method	Specificity (%)	Sensitivity (%)	F1-Score	Accuracy (%)
KNN	73.4	79.0	0.733	72.2
Random Forest	82.9	87.5	0.833	83.1
Bi-LSTM	85.6	84.9	0.849	84
1D-transformer	88.1	90.3	0.891	88.5

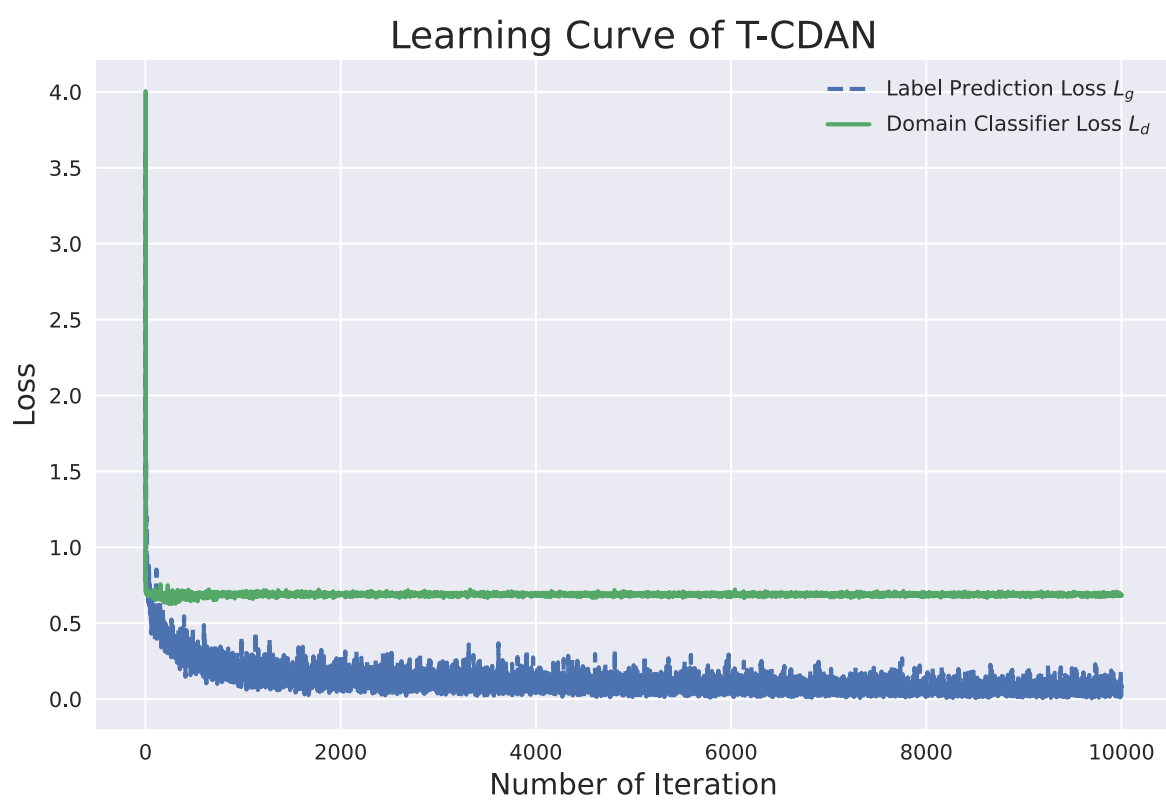


Figure C.1: The learning curve of T-CDAN.

Appendix D Supporting Information for Chapter 5



Figure D.1: The details of the Dragonfly Testing Kit. It includes a sample preparation kit, a test panel (each panel tests five kinds of pathogens), a heating block, and an algorithm-integrated App running on tablets or smartphones.

TH = 0.3	Concentration = 10^3 copies/μL	Concentration = 10^5 copies/μL	Concentration = 10^7 copies/μL
T = 0 min	IC X X X X X X IC IC 	IC X X X X X X IC IC 	IC X X X X X X IC IC
T = 5 min	IC X X X X X X IC IC 	IC X X X X X X IC IC 	IC X X X X X X IC IC
T = 10 min	P P P P P P Y Y 	P Y P P P P Y Y 	P Y Y Y P P Y Y
T = 15 min	P Y P Y P P Y Y 	P Y Y Y Y P Y Y 	P Y Y Y Y Y Y Y
T = 20 min	P Y Y Y Y P Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y
T = 25 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y
T = 30 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y
T = 35 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y

Figure D.2: Algorithm-based detection results for supplementary experiments, $TH = 0.3$. The detected colour on top of each tube: P: pink/negative, Y: yellow/positive, U: unknown, IC: incorrect control, X: not relevant because of control check or empty check failure. T indicates the incubation time since started.

$TH = 0.1$	Concentration = 10^3 copies/μL	Concentration = 10^5 copies/μL	Concentration = 10^7 copies/μL
T = 0 min	IC X X X X X X IC IC 	IC X X X X X X IC IC 	IC X X X X X X IC IC
T = 5 min	IC X X X X X X IC IC 	IC X X X X X X IC IC 	IC X X X X X X IC IC
T = 10 min	P U P P P P Y Y 	P Y U P P P Y Y 	P Y Y Y P P Y Y
T = 15 min	P Y P Y P P Y Y 	P Y Y Y Y P Y Y 	P Y Y Y Y Y Y Y
T = 20 min	P Y Y Y U P Y Y 	P Y Y Y Y U Y Y 	P Y Y Y Y Y Y Y
T = 25 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y
T = 30 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y
T = 35 min	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y 	P Y Y Y Y Y Y Y

Figure D.3: Algorithm-based detection results for supplementary experiments, $TH = 0.1$. Tubes detected as 'Unknown' are circles as shown.

Section D.1: Ethical Approvals

The use of anonymised samples with the Skin Infection Viral Test Panel was approved by the West London National Research Ethics Committee (approval No. 06/Q0406/20). In Burkina Faso, sample collection from symptomatic patients in the Central West Region received the approval from The Comité d'Éthique pour la Recherche en Sante-Burkina Faso (ref: DELIBERATION N°2021-04-084) and the Comité d'Éthique Institutionnel pour la Recherche en Sciences de la Santé of IRSS (N/Réf. A07-2021/CEIRES); the approval for collecting samples from asymptomatic participants was granted by The Comité d'Éthique pour la Recherche en Sante-Burkina Faso (ref: DELIBERATION N° 2024-0361). Written informed consent was obtained from all research participants and/or guardians before recruitment. No ethics were required for the respiratory panel as samples were spiked.

Section D.2: Experimental Details and Results of Supplementary Experiments

To explore the algorithm's response to this abnormal situation and evaluate the method's sensitivity, we carried out supplementary experiments with Skin-tropic virus panels on synthetic DNA (gBlocks, IDT Inc., US). Three concentrations (10^3 , 10^5 , 10^7 copies/ μ L) of all the five pathogen DNA were tested, and photos of the panels were taken at 0, 5, 10, 15, 20, 25, 30, 35 min after incubation started with a smartphone camera (13 MP, HUAWEI P smart 2019, China), to see the colour transforming phases of tubes under various concentrations.

The proposed algorithm flow was applied to the photos taken in the supplementary experiments with the adjustable TH as 0.3 and 0.1 respectively, and the results are shown in Figure D.2 and Figure D.3 (for each tube, P: pink/negative, Y: yellow/positive, U: unknown, IC: incorrect control, X: not relevant because of control check failure). For all concentrations, at 0 and 5 min, no colour changes (including the control tubes) can be observed as the reactions have just begun, and the algorithm correctly identified the abnormality in both figures. As the reactions went on, some tube colours started to transform due to the pH change in the solution, and ambiguous colours appeared for certain pathogens. Whether this transforming status can be captured or not depends on the target's Time-to-Positive (TTP) (which is related to the assay's chemical nature) and the initial concentration. When a moderate decision coefficient $TH = 0.3$ is set, positive reactions can be identified earlier, as illustrated in Figure D.2, indicating the high sensitivity of detection for the algorithm. If stricter decisions are preferred with $TH = 0.1$ (as shown in Figure D.3), some hard-to-identify ambiguous colours will be considered 'in-between' and marked as 'Unknown' by the algorithm. In this case, user

notifications will be pushed by the App to ask for a longer incubation time. As long as the TH is set inside a reasonable range, the algorithm can perform robustly with high sensitivity.

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