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Advancing *in ovo* egg sexing through molecular sex identification of chick embryos using LAMP and RPA assays

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Despite global efforts, minimizing the culling of one-day hatched male chicks remains a critical priority in the poultry industry due to significant socio-economic concerns. To address this issue, various molecular assays have been developed for *in ovo* sex determination, enabling the identification and elimination of male embryos at early developmental stages. However, due to their complexity associated with ensuring high precision, the requirement for advanced infrastructures and the time-intensive nature of current methods, these assays have yet to achieve widespread commercialization. In this study, we developed two novel digital readout assays employing PCR, LAMP and RPA techniques, which were evaluated for sensitivity, specificity and robustness using 82 nine-day-old chick embryos. Our data demonstrate that both newly developed PCR-based assays accurately and reliably determined the sex of all 82 chick embryos. Moreover, the LAMP- and RPA-based assays produced comparable results while offering the advantage of isothermal amplification, enabling detection through naked-eye colorimetric and/or fluorescence-based readouts. Notably, these assays operate within a shorter time frame, with LAMP completing amplification in 20 min at 65 °C and RPA in 30 min at 37 °C. These newly developed assays substantially simplify experimental settings while offering faster and more affordable sexing methods. By addressing critical challenges associated with *in ovo* sexing, they contribute to the advancement of non-invasive *in ovo* sexing techniques, facilitating their potential commercialization in the future.

Keywords PCR, LAMP, RPA, Chick embryos, *In Ovo* sexing

Abbreviations

LAMP	Loop-Mediated Isothermal amplification
RPA	Recombinase Polymerase amplification
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Chd	Chromobox Helicase DNA-binding
Chd-Z	Chromobox Helicase DNA-binding gene on chromosome Z
Chd-W	Chromobox Helicase DNA-binding gene on chromosome W
CHD-F	Female specific fragment of Chromobox Helicase DNA-binding gene on chromosome W
AC18	DNA fragment situated on AC182258 locus
12S	12S ribosomal RNA gene

Background

Large-scale chicken egg production has been increasingly scrutinized in several European and North American countries due to both economic and ethical concerns. In response, efforts to address these challenges have

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primarily focused on optimizing various aspects of the production process, including extending the laying period of hens to reduce replacement rates and transitioning to organic and non-cage farming systems to enhance animal welfare[1]. The culling of one-day-hatched male chicks remains a significant challenge in the poultry industry. To address this issue, two main approaches have been proposed: (i) the use of dual-propose strains, in which male chicks are raised for meat production[2,3]; and (ii) the development of egg sexing technologies[4–6] that enable the identification and elimination of male embryos at early developmental stages. However, despite extensive effort, consumer acceptance of meat from dual-propose breeds remains limited, and existing egg sexing techniques are often complex, time-consuming and require advanced infrastructure[7]. To date, only five sexing techniques have been successfully commercialized[7], highlighting the ongoing need for the development of novel, efficient alternatives.

To date, five major technological approaches have been developed for *in ovo* sex determination of chicken egg, classified based on their biomarker or methodology: (i) volatile organic compounds, (ii) morphological inspection, (iii) acoustic-based methods, (iv) spectral-based methods, and (v) molecular approaches. Among these, molecular-based techniques have garnered significant attention due to their superior accuracy and lower susceptibility to biological variability. These assays typically rely on PCR-based amplification of sex-specific DNA fragments located on either the shared Z chromosome or the female-specific W chromosome in birds[7]. The first PCR-based sexing assay, developed in 1998 used the chromobox-helicase-DNA-binding (*Chd*) gene, which is present on both Z (*Chd*-Z) and W (*Chd*-W) chromosome[8]. While *Chd*-Z and *Chd*-W share conserved sequences at their extremities, *Chd*-W contains a female-specific region, allowing for sex discrimination using a single primer pair. This method enable differentiation between male embryos (ZZ, *Chd*-Z only, producing a smaller amplicon) and females (ZW *Chd*-Z & *Chd*-W, yielding a larger amplicon) and has been successfully applied across various avian species[9–12]. Over time, refinements in molecular sexing techniques, including the identification of new genetic targets[13,14], the introduction of multiplex PCR[15], and the adoption of high-throughput real-time PCR[16] have significantly improved both the speed and accuracy of these assays. However, the practical application of these methods remains constrained by their reliance on complex amplification technologies, which require sophisticated instrumental facilities (thermocyclers, quantitative thermocyclers, and gel/capillary electrophoresis) and specialized infrastructure. These limitations hinder their scalability and broader implementation in commercial poultry production.

To overcome these limitations, a range of isothermal DNA amplification techniques have emerged in recent years, offering potential solutions to reduce the reliance on complex laboratory infrastructure. These methods operate at constant and lower temperatures (37–65 °C) compared to cyclic thermal fluctuations required for conventional PCR, making them more accessible and practical for various applications. Among these techniques, LAMP has gained significant attention in molecular diagnostics and is increasingly being recognized as a viable alternative to conventional PCR[17–20]. LAMP has been widely adopted due its high sensitivity and rapid turnaround time, typically producing results in less than 30 min. This efficiency is attributed to its use of multiple primer sets (three sets) and the Bst DNA polymerase, which possesses strong strand displacement activity[20]. Furthermore, LAMP is compatible with various detection methods, ranging from conventional gel electrophoresis to fluorescence-based and colorimetric (naked-eye) readout. Notably, the combination of naked-eye observation with digital binary output (Yes/No readouts) has facilitated the development of point-of-care diagnostic tools for the onsite detection of infectious diseases such as malaria[21], dengue[22] and COVID-19[23,24]. Similarly, RPA operates at significantly lower temperature (37–42 °C) thanks to the use of DNA recombinase enzyme, allowing for rapid detection and simple visualization of amplified products[25,26]. Importantly, as RPA reactions occur within a temperature range compatible with egg incubation condition, this technique presents a promising approach for *in ovo* sex determination within poultry production systems, enabling onsite implementation with minimal infrastructure requirements.

In our previous work[9], we developed a duplex PCR and a quantitative PCR (qPCR) assays using SWIM/12S and DMRT/Xho-I markers, respectively, to enable specific, rapid and robust sex determination of 9-day-old chicken embryo. In this work, we expanded upon the previous work by employing LAMP and RPA to develop two novel assays based on two newly designed sex-specific DNA markers. First, we identified two new sex-specific DNA fragments: one within the well stablished *Chd-W* gene, and another within the *AC182258* locus (AC18), both of which are located solely on the female-specific W chromosome. Second, we demonstrated that fluorescent LAMP could detect AC18 fragment, enabling sex discrimination in under 20 min at constant temperature (65 °C). This approach is compatible with naked-eye colorimetric detection and scalable for high-throughput applications. Finally, we demonstrated that RPA allows for sex determination at 37 °C in under 30 min, significantly reducing both assay complexity and associated costs. Additionally, we showed that our methods eliminate the need for DNA input normalization, further simplifying the experimental workflow and enhancing feasibility for large-scale implementation in the poultry industry.

Results

PCR validation of two newly developed sexing assays for chicken embryos

To design two novel sexing assays, we targeted two distinct genomic loci on the female-specific W chromosome: the *Chd* gene and the *AC182258* locus. Primer sets were screened to amplify DNA fragments ranging from 180 to 220 bp (Table S1). Notably, unlike previous CHD-based PCR sexing methods that relied on size discrimination between *Chd*-Z and *Chd*-W, our approach specifically targeted a region unique to *Chd*-W (female), absent in *Chd*-Z (male). Various PCR primer combinations were evaluated for their selectively amplify female-specific fragments (here referred to as CHD-F and AC18). In the majority of primer sets tested, CHD-F (Figure S1A) and AC18 (Figure S1B) produced clear, single bands for females, with no amplification observed in male samples on agarose gel. Nonetheless, compared to CHD-F, AC18 design demonstrated slightly lower efficiency as several primer combinations resulted in false–positive amplifications (Figure S1B).

Following the selection of the optimal primer pairs for each assay (F3R1 for CHD-F; F2R1 for AC18, Table S1), their performance was evaluated on 82 samples of 9-day- old chick embryos (ISA Brown eggs, *Gallus gallus*). To this aim, we used genomic DNA extracted from dissected brain tissue of chick embryos as a template for the PCR reactions[9]. Quantification of purified DNA revealed a wide range of DNA concentrations among the 82 samples, varying from 1 ng/µL up to 226 ng/µL (Figure S2, Table S2). Although statistical analysis using the Kolmogorov-Smirnov test indicated a normal distribution of DNA concentrations (p-value 0.08), approximately 21% of the samples exhibited significantly low DNA yields (< 10 ng/µL). Despite this variability, no DNA concentration normalization was performed in the subsequent experiments to evaluate the robustness of the assays.

For both CHD-F and AC18 assays, an internal control targeting *12 S* rRNA gene[9] was incorporated alongside the female-specific target sequences, resulting in the CHD-F/12S and AC18/12S assay designs. PCR amplification using CHD-F/12S assay with the selected F3R1 primer pair (Table S1) was first performed. Capillary electrophoresis (Labchip) analysis of the PCR products showed two distinct groups based on the number of amplicons. In the first group (40 samples), a single band corresponding to *12 S* amplicon (139.9 ± 0.9 bp) was detected, indicating a male genotype (Fig. 1A, B). The second group (42 samples) exhibited two distinct bands: the *12 S* amplicon (142.2 ± 0.2 bp) and an additional band of 179.2 ± 0.2 bp, which corresponded to the predicted CHD-F target fragment, confirming a female genotype (Fig. 1A&C).

At this stage, we predicted that Group 1 corresponded to male samples, while Group 2 represented female samples. For both *12 S* (Figure S3A) and CHD-F (Figure S3B) amplicons, no correlation was observed between concentrations of extracted DNA and the amplified PCR products measured by LabChip. However, a statistically significant difference in 12 S amplicon concentration was observed between the two groups (p-value < 0.01), with the predicted female group exhibiting lower *12 S* concentrations (Figure S3A). Notably, one sample (F2) exhibited lower *12 S* amplification (Figure S3A), while three samples (F1, F2 and F3) exhibited lower amplification for the CHD-F target (Figure S3B).

To further evaluate the reliability of our approach, PCR amplification using the AC18/12S assay with the selected F2R1 primer pair (Table S1) was performed on the same DNA sample set. Similar to CHD-F assay, capillary electrophoresis revealed two distinct groups: one comprising 40 samples containing only a single *12 S* amplicon (139.8 ± 1.0 bp) and another consisting of 42 samples displaying two distinct amplicons corresponding

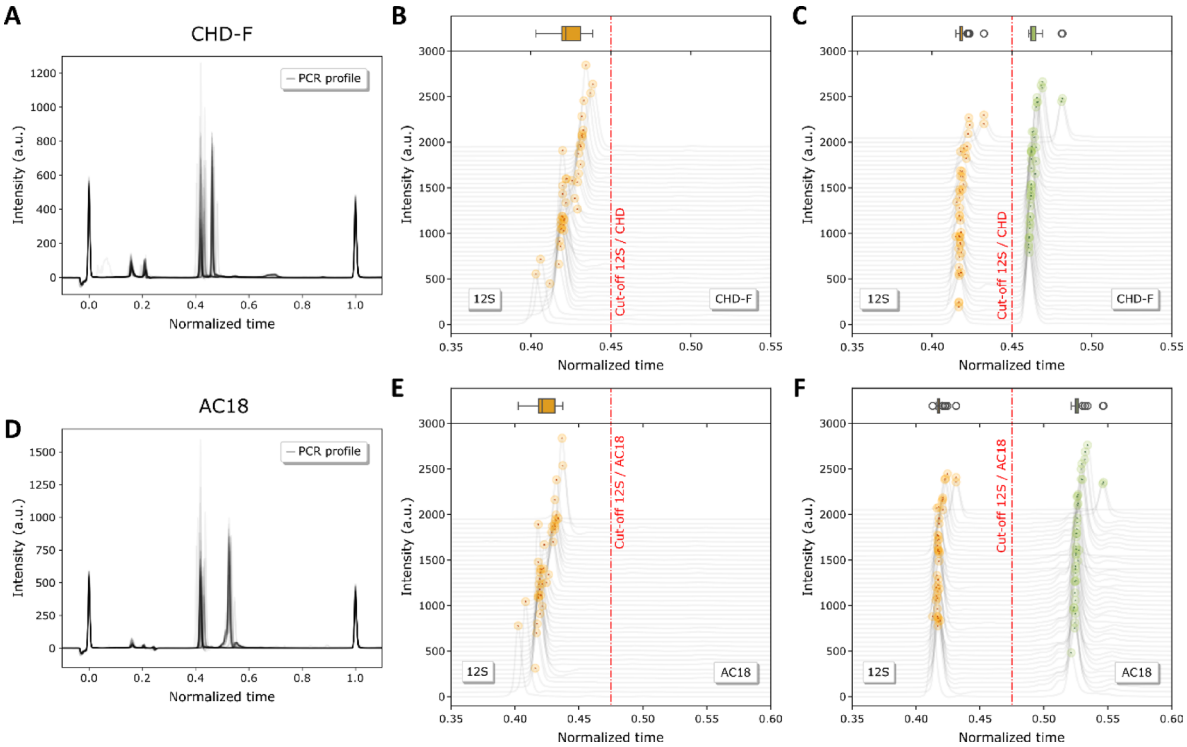

Fig. 1. Validation of two novel PCR assays for sex determination in 82 chicken embryos. Capillary electrophoresis results for the CHD-F assay (**A-C**) and AC18 assay (**D-F**) demonstrate the capacity of both methods to accurately differentiate male from female samples. **(A)** Superimposed electropherograms of 82 samples analysed by 12 S/CHD-F assay, **(B)** Electropherograms of male samples, displaying only the *12 S* amplicon., **(C)** Electropherograms of female samples, exhibiting both the *12 S* and CHD-F amplicons. **(D)** The superposition of the 82 electropherograms analysed by 12 S/AC18 assay. **(E)** Electropherograms of male samples containing only the *12 S* amplicon. **(F)** Electropherograms of female samples containing both *12 S* and AC18 amplicons. The 12 S amplicons are shown in orange, while female-specific target amplicons (CHD-F and AC18) are shown in green. In panels B, C, E and F, samples are arranged in increasing order of normalized *12 S* peak time rather than by the reference order.

to the *12 S* fragment (142.0 ± 0.2 bp) and the AC18 target (229.3 ± 0.2 bp) (Fig. 1D, E and F). As expected, classification based on the AC18 assay was identical to that obtained with the CHD-F assay, demonstrating 100% concordance between the two assays (Table S2). Similar to previous results, no correlation was observed between the extracted DNA concentration and amplification efficiency of either the *12 S* or the AC18 amplicons. However, the *12 S* amplicon concentration was significantly lower in the predicted female group compared to the male group (p-value < 0.01) with the sample F2 again displaying significantly low *12 S* amplification (Figure S4A). For the AC18 target, we again noticed reduced amplification (up to 43-fold) in samples F1, F2 and F3 (Figure S4B).

To confirm the accuracy of sex identification by the two novel assays, the DNA sample set was further analyzed using our previously established SWIM/12S assay[9]. PCR product analysis (Fig. 2) validated the correct sex classification of all the 82 samples, identifying 40 male samples that exclusively amplified the *12 S* amplicon (146.7 ± 0.2 bp, Fig. 2B) and 42 female samples that produced both the *12 S* and SWIM fragments (148.9 ± 0.3 bp and 231.2 ± 0.3 bp, respectively) (Fig. 2C). Consistent with previous results, no correlation was observed between the DNA concentration and amplification efficiency for either *12 S* or SWIM amplicons (Figure S5). Unlike the CHD-F/12S and AC18/12S assays, the SWIM/12S assay did not exhibit reduced *12 S* amplification in the duplex PCR configuration. Indeed, no significant difference in amplicon concentration was observed between *12 S* and SWIM targets (p-value = 0.18). However, the *12 S* concentration in sample F2 was approximately two-fold lower than the group mean, while SWIM amplification was up to six-fold lower in samples F1, F2 and F3 (Figure S5). Furthermore, the sex validation using the SWIM marker revealed that female samples had significantly higher extracted DNA concentrations (84.7 ± 8.5 ng/µL) compared to male samples (57.9 ± 10.2 ng/µL) (Figure S2).

Loop-Mediated isothermal amplification (LAMP) assay for rapid sexing of chick embryos

To enhance the suitability of our assays for field applications, we adapted the two novel PCR-based sexing assays to LAMP, which enables amplification at a constant temperature. As such, various LAMP primer sets (Table S1) were designed and validated using endpoint colorimetric LAMP (Figure S6) for their ability to detect female-specific sequences. Results showed that both target candidates, CHD-F and AC18, successfully enabled sex discrimination via naked-eye colorimetry, with assay V for CDH-F and assay D and G for AC18 demonstrating effective differentiation. For large-scale validation, the primer pair of assay D for AC18 was evaluated in the previously assessed set of 82 chick embryo samples using fluorescent LAMP. The assay demonstrated high robustness, correctly identified the sex of all samples except for the sample F1 (Table S2). In particular, the initiation of the exponential amplification (Ct value) for female samples remained consistent (13.5 ± 0.2 min), except for samples F2 and F3, which exhibited significantly delayed amplification (19.0 ± 1.1 min) (Fig. 3A and B). Although minor variations in fluorescence intensity and Ct values were observed, no correlation was observed between extracted DNA concentration and Ct values (Fig. 3C). For male samples and negative controls, no amplification was observed within the 40-minute reaction time, confirming the specificity of the assay. The AC18 LAMP assay hence provides a binary (digital) readout, reliably distinguishing samples based on the presence or absence of the female specific AC18 target on chromosome W.

While digital responses of the LAMP assay facilitate straightforward data interpretation, the absence of an internal control prevents verification of enzymatic activity within the reaction. Given the high tau value observed for the synthetic AC18 plasmid (Fig. 3A), we incorporated it as an internal control to ensure proper enzymatic function. To this aim, a spike-in experiment was conducted by adding varying concentrations of AC18 plasmid to a random male (M1) and female (F37) sample prior to amplification. As expected, increasing plasmid concentration resulted in stronger detection signal for the non-template negative control and male sample, with a Ct value as low as 27.1 at 10 ng/µL (Figure S7). On the contrary, plasmid spike-in did not affect the

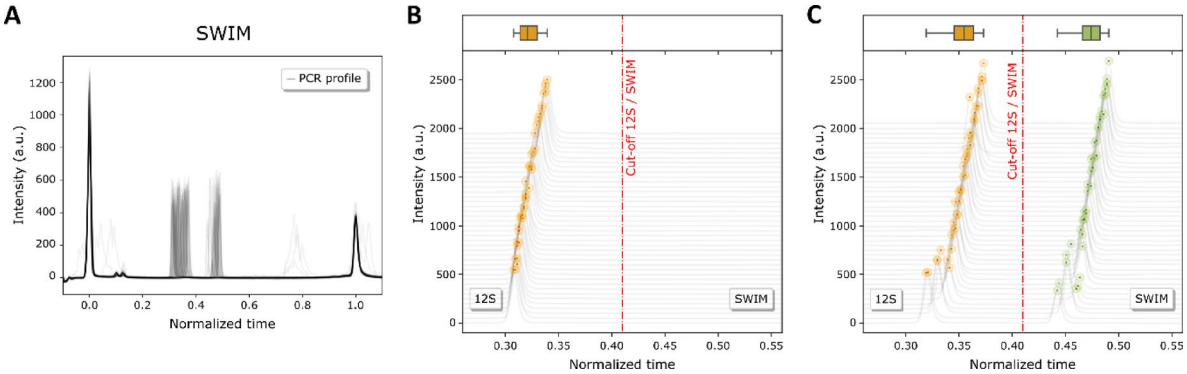

Fig. 2. Validation of sex identification in 82 chick embryo samples using the SWIM/12S assay. Capillary electrophoresis results for the previously established SWIM/12S assay confirm the accuracy of sex determination across 82 chick embryo samples **(A)** Superimposed electropherograms of all 82 samples. **(B)** Electropherograms of male samples, showing amplification of the *12 S* amplicon only. **(C)** Electropherograms of female samples, exhibiting amplification of both *12 S* and SWIM amplicons. The *12 S* amplicons is represented in orange, while SWIM amplicon is shown in green. In panels B and C, the samples are arranged in increasing order of normalized *12 S* peak time rather by reference order.

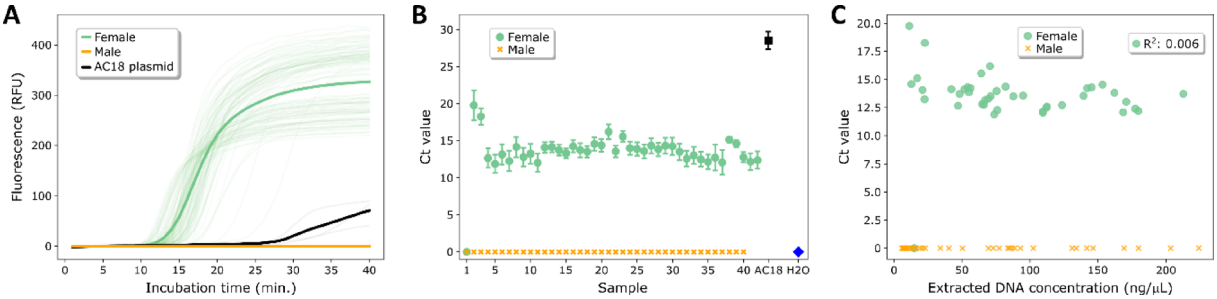

Fig. 3. Rapid sex identification of chick embryos using the AC18 LAMP assay. **(A)** Real-time fluorescence curves of AC18 LAMP assay for the 82 chick embryo samples and the positive control (Plasmid containing AC18 fragment). **(B)** Ct values (initiation of the exponential amplification) plotted against sample reference names. **(C)** Ct values as a function of extracted DNA concentration.

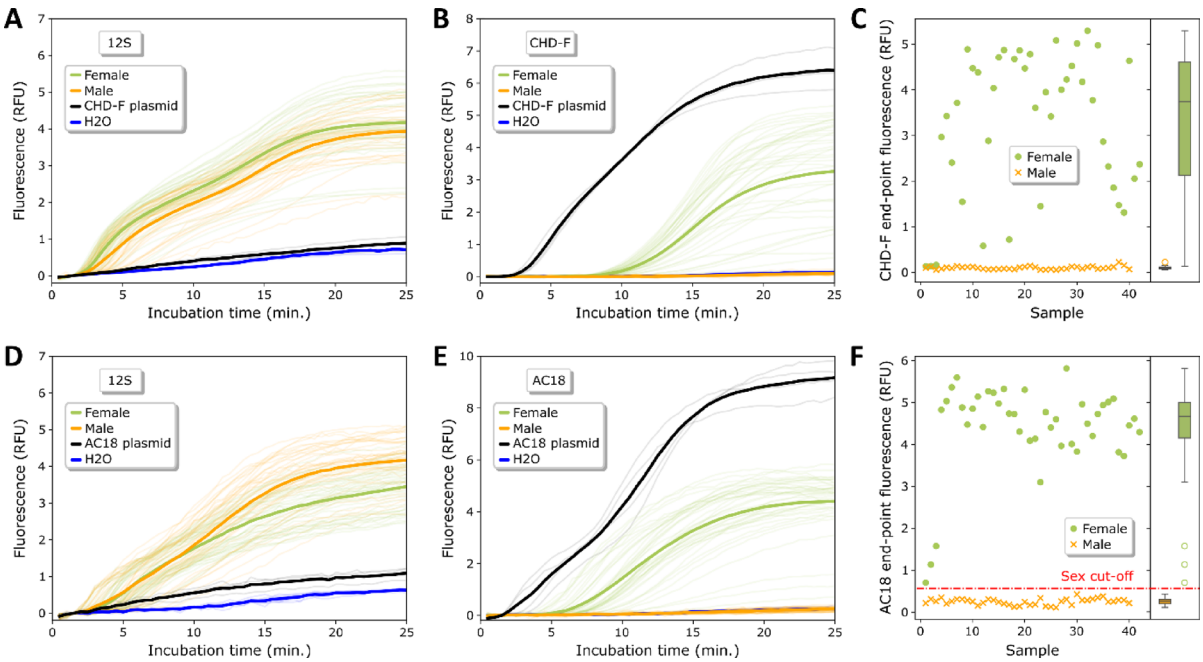

Fig. 4. RPA assays for sex identification of chick embryosat 37 °C. Real-time fluorescence analysis of the CHD-F/12S RPA assay for the simultaneous amplification of **(A)** the *12 S* locus and **(B)** the CHD-F fragment, followed by **(C)** end-point fluorescence measurements of panel B. Real-time fluorescence analysis of the AC18/12S assay is shown for **(D)** the *12 S* fragment and **(E)** the AC18 fragment with **(F)** end-point fluorescence measurement of panel E.

Ct value of the female sample, maintaining a ΔCt of 17.3 (Male vs. Female), thereby allowing clear differentiation between male and female samples.

Recombinase polymerase amplification (RPA) assays for sexing of chick embryos at low temperatures

To further reduce the dependency on advanced laboratory infrastructure and minimize costs, we adapted the CHD-F and AC18 assays to RPA, which operates at 37 °C. First, various RPA primer sets (Table S1) were designed and validated using the endpoint RPA assay (Figure S8). The most efficient primer pairs (Pair 5 for CHD-F and Pair 27 for AC18) demonstrated amplification performance comparable to their PCR-based counterparts (Figure S8). These primers were subsequently validated in a large-scale data set of 82 samples using fluorescent RPA-exo assays, where 12 S primers (amplified both male and female samples) were included as an internal control in a duplex configuration (Fig. 4).

The CHD-F/12S fluorescent assay showed consistent exponential amplification of the *12 S* target in all 82 samples. Moreover, end-point fluorescence intensity for *12 S* amplification did not significantly differ between male and female samples (p-value = 0.10) and was independent of the extracted DNA concentration (Figure S9A). In contrast, exponential amplification of the CHD-F fragment was observed exclusively in female samples

(Fig. 4B), except for F1, F2 and F3, which exhibited reduced signal intensities (Fig. 4C). Again, no correlation was found between DNA concentration and end-point CHD-F fluorescence signal (Figure S9B). Similarly, results obtained with the AC18/12S assay were consistent with those from the CHD-F/12S assay. The *12 S* fragment was amplified in all 82 samples, with signals unaffected by either DNA concentration or sex identity (Fig. 4D and S9C). Additionally, AC18-specific amplification was observed exclusively in female samples, while all male samples remained negative (Fig. 4E). End-point fluorescence analysis revealed that female samples had an average signal intensity of 4.40 ± 0.17 RFU, which was 18-fold higher than that of male samples (0.25 ± 0.01 RFU), allowing the establishment of a sexing cut-off value at 0.56 RFU. However, signals from samples F1, F2 and F3 remained significantly lower than the average female sample) (Fig. 4F). Once again, no correlation was observed between DNA concentration and end-point AC18 fluorescence (Figure S9D). To assess the impact of DNA concentration RPA performance, we conducted amplification reactions using varying DNA input volumes from highly concentrated samples (M4: 204.7 ng/µL and F17: 214.3 ng/µL) and compared them to a low-concentration female sample (F12:16.5 ng/µL). Fluorescent measurement revealed that amplification curves for both *12 S* and AC18 remained identical across different DNA input volumes in samples M4 and F12. However, significant impairments in RPA amplification were observed for F17 sample (Figure S10). This impairment is more pronounced for AC18 than for *12 S*, indicating that the AC18 assay remains effective across a DNA concentration range of 0.13 ng/µL up to at least 25.7 ng/µL.

Finally, to further evaluate the feasibility of low-temperature amplification, we tested the AC18/12 assay at room temperature (25 °C) (Figure S11). As expected, a longer incubation time (50 min) was required for both *12 S* and AC18 RPA reactions to reach the plateau signals. However, under these conditions, the assay failed to detect samples F1, F2 and F3. Despite this limitation, all other female samples were correctly identified, exhibiting fluorescence signals approximately three times higher than those of male samples. These results demonstrate that the AC18 assay maintains a significant performance for sex discrimination (P-value < 0.01) even at 25 °C.

Discussion

The culling of one-day hatched male chicks remains a major socioeconomic and ethical challenge in the poultry industry. Various *in ovo* molecular sexing techniques have been developed to address this issue[4–6]. However, their adoption is hindered by technical complexity, dependence on advanced infrastructure, and high financial/time costs. In this study, we designed two novel digital-response assays and adapted them for LAMP isothermal amplification for its rapidity and RPA, enabling rapid and simplified sex determination. Furthermore, our assays do not require DNA input normalization, further simplifying the workflow and improving practical applicability.

Conventional molecular assays typically require DNA concentration normalization to ensure reliable amplification and consistent output[9]. Our analysis of 82 chick embryo samples revealed up to a 226-fold difference in extracted DNA concentration (Figure S1), likely due to variations in sample handling, conservation and input material (brain tissue) quantities. To assess the robustness of our assay, we eliminated the DNA normalization step, using fixed volume of extracted DNA rather than adjusting for concentration. Despite a broad range of DNA input concentration (3-678 ng), all three genetic markers (*12 S*, CHD-F, AC18) were successfully amplified in all samples, demonstrating PCR-based assay resilience. Importantly, amplicon intensity was independent of DNA concentration (Figure S3-S5), suggesting that PCR reactions reached saturation in all cases. Our previous study showed that 1 ng of DNA is sufficient to achieve PCR plateau amplification in under 30 cycles for female samples and 40 cycles for male samples[9].

To further simplify *in ovo* molecular, we transitioned from analogue (quantitative) readout to digital (Binary Yes vs. No) outputs. This shift is crucial as it reduces the expertise required for data interpretation and the need of specialized analytical instrumentation. Conventional CHD-based PCR assays rely on size differences between *Chd*-W (female-specific) and *Chd*-Z (shared) amplicons, requiring high-resolution electrophoresis or capillary analysis for sex determination[8–12]. These size differences can vary between 11 bp and 165/179 bp, requiring careful downstream analysis. In contrast, our CHD-F and AC18 assays allow for straightforward sex determination based on the presence or absence of the amplicon (Fig. 1C and F), significantly simplifying the process. In particular, we observed that female samples exhibited lower 12 S amplification compared to male in CHD-F/12S and AC18/12S duplex assays. This could be attributed to the competition between targets during co-amplification, slightly reducing the efficiency of both. Therefore, omitting the *12 S* internal control could further enhance CHD-F and AC18 amplification, further simplifying sex discrimination process. Collectively, these findings confirm that our PCR assays achieve 100% accurate sex identification without requiring DNA input normalization, making them highly scalable for industrial applications.

For large-scale *in ovo* sexing, rapid and high throughput approaches are essential[1]. To address this need, we adapted our assays to LAMP, achieving sex determination within 20 min with binary digital readouts. Colorimetric LAMP (Figure S6) enables visual detection with minimal equipment (only a 65 °C incubator) (Table 1), paving the way for portable sexing devices[27]. However, manual interpretation of colorimetric results limits automation, posing challenges for high throughput applications. To overcome this challenge, we adapted fluorescence-based LAMP, enabling real-time monitoring in a small reaction volume (12.5 µL). The AC18 fluorescent LAMP assay correctly sexed 81 out of 82 samples, confirming its reliability (Fig. 3). While real-time fluorescence LAMP is generally used for quantitative analysis, its continuous amplification process is highly prone to stochastic variations at low template concentrations, leading to digital-like outputs rather than true quantitative results[19]. Given that sex determination relies solely on the presence or absence of the female-specific targets, fluorescent LAMP assay does not require high–resolution quantification, making it well-suited for large-scale industrial applications. Additionally, the inclusion of 0.5 ng/µL of AC18 plasmid an internal control ensures reliable enzymatic verification in LAMP reactions.

In addition to high accuracy and simple implementation, cost effectiveness is a critical factor for the industrial application of sexing assay due to its significant economic implications. While our calculations indicated that

Items	PCR	LAMP	RPA
Detection method	Labchip	Sybr green /colorimetry	Probe
Steps required	2	1	1
Time (minutes)	60 + 60	20	25
Machines required	Thermocycler & Labchip	Incubator (65 °C) & fluorometer	Incubator (37 °C) & fluorometer
Power consumption (W)	240 (94 °C)	150 (65 °C)	110 (37 °C)
Estimated consumable cost per reaction (€)	2.70	2.21	2.26
Accuracy	+++	+++	+++

Table 1. The performance, cost and efficiency of the three molecular techniques used in this study. Power consumption has been measured at the maximum temperature required by the assay. Details of estimated consumable cost per reaction are showed in table S3.

the consumable costs per reaction were comparable between PCR and LAMP assays, the LAMP assay reduced incubator power consumption by 38% (Table 1), excluding the additional energy required for the Labchip analysis. Furthermore, our RPA-based sexing assay, operated at 37 °C, demonstrated even greater cost effectiveness, with 20% reduction in consumable costs and 58% lower power consumption than PCR. RPA assay results confirmed the CHD-F assay's ability to identify sex status 96% sensitivity (79 out of 82 samples) and a 100% specificity, while the endpoint AC18 assay displayed both 100% sensitivity and specificity, with a clear defined cut-off value (Fig. 4C). Notably, RPA assays can also generate digital readouts (i.e. the presence or absence of amplification), making them highly suitable for streamlined analysis. However, the irregular shape of RPA amplification curves (Fig. 4A) posed a challenge for the commercial software, which struggled to accurately determine the onset of exponential amplification using the second derivative method. Consequently, a thresholding approach was found to be more appropriate. Interestingly, we observed that in female samples, the final fluorescence intensity decreased as the input DNA concentration increased (Figure S10), likely due to an imbalance between the target and primer concentration. However, beyond a certain input threshold, the fluorescence level stabilized, maintaining sufficient intensity for reliable sex identification. This finding highlights the robustness of the assay, even in the presence of variable DNA inputs. To further explore the potential for onside applications, we tested the AC18/12S RPA-exo assay at room temperature (25 °C). A slight decrease in assay robustness was observed, as samples F1-F3 failed to achieve exponential amplification within 50 min. This outcome is consistent with the reduced amplification efficiency observed for these samples at 37 °C, likely exacerbated by the inherent temperature sensitivity of RPA[26]. While other female samples also exhibited reduced performance at 25 °C, with the fold change between female and male fluorescence signals dropping from 18-fold to 3-fold, they were still correctly identified, demonstrating the assay's resilience to temperature-induced impairments.

Throughout the study, three samples (F1, F2 and F3) consistently exhibited significantly lower PCR amplification efficiencies for the target loci (Figure S3-S5), with distinct amplification behaviour observed. On the one hand, sample F2 consistently showed the lowest *12 S* amplification. Despite being the female sample with the lowest extracted DNA concentration, 12 male samples with even lower DNA concentrations displayed higher *12 S* amplicon intensity during simplex amplification. This suggests a potential partial impairment of both targets in the duplex amplification configuration. On the other hand, samples F1 and F3 exhibited normal *12 S* amplifications but reduced amplification of the female specific targets. The hypothesis that low DNA input hinder target amplification was ruled out, as F1 (10.3 ng/µL) and F3 (18.3ng/µL) had higher DNA concentrations than other female samples (e.g., F39 = 8.1 ng/µL, F12 = 16.5 ng/µL and F10 = 18.1 ng/µL), which achieved robust female target amplification. These findings suggest that variability in target copy number across samples may significantly impact PCR results, even when total extracted DNA concentration is relatively low. Despite these challenges, the PCR assays successfully identified all three samples (F1, F2, and F3) as female, demonstrating their robustness in chick embryo sexing. Interestingly, while sample F2 showed the lowest amplification efficiency in PCR assays, F1 displayed the least efficient amplification in RPA and failed to amplify in LAMP assay. For samples F2 and F3, although lower amplification efficiencies were observed, sex determination remained feasible using AC18 LAMP and RPA assays, further supporting their reliability across different amplification methods.

Conclusions

This study established two novel molecular assays (CHD-F and AC18) for the accurate and scalable sex determination of 9-day-old chick embryos, offering a binary digital readout for simplified interpretation. We first validated their performance using PCR, followed by their adaptation to LAMP, reducing reaction time to under 20 min. Finally, we demonstrated the feasibility of RPA at 37 °C, minimizing both experimental complexity and cost. We envision that these two novel assays (CHD-F and AC18) combined with the adapted amplification techniques, present a transformative potential for the poultry industry across different production scales. The rapid and high throughput nature of LAMP assays enables efficient and user friendly large-scale *in ovo* sexing. Meanwhile, the lower temperature requirement for RPA assays provides a simple, cost-effective and field-compatible solution for *in ovo* sexing in industrial poultry production[28]–[29]. These advancements contribute to enhancing economic, ethical and environmental sustainability in the poultry industry, facilitating the transition toward automated and commercialized sexing technologies.

Materials and methods

Eggs incubation

Fertilized ISA Brown eggs were purchased from a commercial supplier (SFPA, Saint-Marcellin, France), stocked at 19˚C for a maximum of 10 days after laying and before incubation. Eggs were incubated in a dedicated incubator (Masson, Soyans France) at 37.5˚C, 55% humidity and tilted every hour. Incubation was arrested at day 9.

Sample collection and Lysis

10–20 mg of brain tissue from day 9 post-hatching embryos of 82 ISA Brown eggs was recovered and treated as previously described[9]. In brief, collected samples were lysed for 3 H at 55 °C in 150 µl of lysis buffer containing 10% of chelating beads (Chelex 100 Biorad), 0.2% SDS, 10mM Tris-HCl pH8, 0.2 µg/µl Proteinase K, followed by a denaturation step at 95 °C for 15 min. Supernatant of lysate samples was recovered by centrifugation for 5 min at 13,000 g at room temperature and submitted to DNA purification using QIAamp DNA mini kit (Qiagen 51306). DNA quantification was performed post-purification using Nanodrop One (Thermo Scientific, Wilmington USA). All samples were stored at -80˚C until used.

Primer design

Primers for all sex determination assays were designed based on the two female specific sequences located on the female-specific W chromosome: *Chd*-W (Chromobox-Helicase-DNA-binding, GenBank Accession No GU132944.1) and *AC182258* locus (GenBank Accession No AC182258.2). Blasting between *Chd*-W and *Chd*-Z was performed to identify specific female fragments (CHD-F) (Table S1). The un-matched fragment situated from position 151–326 of the *Chd* gene would allow female identification as it is present only on female-specific W chromosome, hence, primers were designed based on this identified fragment (Table S1). Regarding the *AC182258* locus, we used a fragment situated on the female-specific W chromosome from position 6942–7267 for designing the AC18 primers with various amplicon lengths (Table S1). Primers for the internal control were designed based on the 12 S ribosomal RNA gene (Accession No MF041985.1).

BIP, FIP and loop primers for the LAMP assay were designed using PrimerExplorer V5. Both primers had been consciously designed to be compatible with the RPA, following TwistDx guidelines regarding primer and amplicon length. For real-time measurement of RPA amplification assisted by exonuclease, corresponding hydrolyse probes for each assay were designed (Table S1) following the instructions of TwistDx. Briefly, RPA probes contain a long amplicon specific sequence (about 45 bp) on to which a fluorophore (FAM or TAMRA) and a quencher (BHQ-1) were incorporated. A basic nucleotide analogue (tetrahydrofuran, THF) residue was introduced in between the fluorophore and the quencher as the recognition site for the exonuclease, whose activity will degrade the probe and separate the fluorophore from the quencher.

PCR amplification and analysis

PCR reaction mix was prepared in 96 well-plate using 5 µL of 2X Master Mix (Invitrogen Platinum Green Hot Start PCR) and 200 nM of primer set SWIM/12S, CHD-F/12S or AC18/12S at a ratio 2:1. 3 µL (constant volume) of purified embryo DNA was added into each well. Amplification was performed on peqSTAR 96X thermocycler (Ozyme, Montigny-le-Bretonneux, France) as previously described[9]. In brief, one initial denaturation at 94˚C for 2 min followed by 35 cycles comprising of: (1) 30 s. at 94˚C for the denaturation, (2) 30 s at 55˚C for the annealing, and (3) 30 s at 72˚C for the elongation. A final extension cycle at 72˚C for 5 min was performed. Negative Control (Non – template) were performed for each experiment. Sex determination was performed using two reference PCR assays SMIW/12S[9] and CHD 2250 F/2718R[30]. All samples were analysed by two novel PCR assays AC18 and CHD-F/12S. Negative (non-template) and positive controls (synthetic plasmid containing the target amplicon, purchased from IDT) were added for each isothermal reaction. Real-time qPCR thermal cycling reactions were performed by the LightCycler 480 (Roche, Meylan France) directed by the LightCycler 480 Software (version 1.5.1.62).

LAMP sexing assays

Reaction mix for fluorescent LAMP assays were prepared on ice using WarmStart LAMP Kit (NEB, E1700) and NEB LAMP fluorescent dye (NEB B1700S). A premix primer solution containing 16 µM of FIP and BIP primers and 2 µM of AC18 primers was prepared prior to the reaction mix preparation. For one reaction, 1.25 µL of the primer mix solution was added to 6.25 µL of 2X NEB LAMP Master Mix, 0.125 µL of 50X NEB LAMP dye and 1.875 of H_2O. The reaction mix was distributed on a 348 well-plate (Axygen) followed by the addition of 3 µL extracted DNA sample. LAMP amplification was carried out at 65 °C for 40 min using the LightCycler480 (Roch, Meylan, France) with one fluorescent acquisition every 30 s. For each experiment, negative control (water) and positive control (plasmid) were performed. The samples were assessed in triplicate. For colorimetric LAMP assessment, the WarmStart colorimetric LAMP master mix (M1800S) was used, following the recommended protocol (M1800).

RPA sexing assays

Endpoint RPA amplification reactions were performed as recommended by the manufacture manual (TwistAmp Liquid basic RPA kit, Ref TALQBAS01). In brief, a pre-master mix for each reaction was prepared on ice with 25 µL of 2X buffer, 5 µL 10X E-mix, and 9.2 µL H_2O containing 1.8 mM of total dNTPs following by vortexing and a brief spin. 2.5 µL of core reaction mix was then added to the lid of the tube containing the pre-master mix. The 41.7 µL of complete master mix was homogenised by 10 full inversions of the tube, followed by a brief spin. In another tube, on the lid, three reagents were added separately in their different corners including 2.4 µL of 10 µM of each primer, 2.5 µL of MgOAc and 1 µL template DNA. The reaction preparation was finally completed by the

addition of 41.7 µL master mix at the bottom of the tube. RPA reaction was activated by spinning all the materials from the lid. Amplification was carried out at 37 °C for 30 min on a thermoblock (Eppendorf).

Real-time RPA amplification reactions were performed at 37 °C for 30 min as described in the manufacture's manual instruction (TwistAmp™ exo Kit, Ref TAEXO02KIT) with a smaller volume adjusted to use the Roche LightCycler 480. Briefly, a pre-reaction mix for each reaction was prepared on ice with 2 µL of the primer mix at 10 µM (ratio 2:1 of sexing target to 12 S target), 0.6 µL of 10 µM exo probe mix, 29.5 µL of the Primer Free Rehydration buffer and 9.4 µL H_2O. The pre-reaction mix (41.5 µL) was then used to resuspend one lyophilized TwistAmp® exo reagents. The complete reaction mix was homogenised by pipetting. On each well of a 384 well-plate (Axygen), a drop of 3 µL sample (0.5 µL of purified embryo DNA + 2.5 µL H_2O unless otherwise stated) was added to the upper part of the well. In the same well, another drop of 1.25 µL MgOAc (280 mM) was added onto the opposing side of the well's wall followed by the addition of 20.7 µL of the complete reaction mix. Following a brief vortex and centrifugation, real time RPA reactions were performed at 37 °C on the LightCycler480 (Roch, Meylan, France) for 25 min with one acquisition of fluorescent signal every 30 s or at 25 °C on the CFX Opus Real-Time PCR System for 50 min with an acquisition every 42 s. For each sexing reaction, water negative controls and positive controls (plasmid consist of target sequence) were added.

Endpoint product analysis by agarose gels and capillary electrophoresis

Amplification products were loaded on a 2% Gelgreen (Biotium, California USA) stained 1.5% agarose gel in 0.5X TAE buffer and separated by electrophoresis at 100 Volts for 30 min. 1 µg of DNA ladder (GeneRuler 50 bp or GeneRuler 100 bp, Fisher Scientific, Illkirch France) served as the reference for the migration. Gels were revealed by Blue LED GelPicBox at 430 nm (Nippon Genetics, Düren Germany).

Capillary electrophoresis (Capillary LifeSciences, France) controlled by the Labchip GX version 4.1.1619.0 SP1 software was performed using 10 µl of the amplification products. The time of the capillary electrophoresis results was normalized by a home-made routine code using two internal labchip markers, a lower maker at the beginning of the gel, and an upper maker at 1500 bp and 7000 bp for ladder 1 K (for SWIM) and ladder 5k (for CHD-F and AC18), respectively.

Data analysis

Home-made Python codes (version 3.10.4) were developed to automate data analysis. Regarding the labchip data, each electropherogram profile had baseline removed prior to time normalization using the two extreme markers (Fig. 1A and D). The LAMP and RPA real-time data baseline was removed at the early time points. Scipy version 1.14 was used do statistical analysis, where Mann-Whitney U (scipy.stats.mannwhitneyu) with asymptotic method (i.e. p-value calculated by comparing to normal distribution and hence correcting for ties) was used. All statistical calculations show the mean values ± standard error of the mean.

Data availability

Data is provided within the manuscript and its supplementary information files.

Received: 6 January 2025; Accepted: 21 July 2025
Published online: 29 July 2025

References

1. Gautron, J., Réhault-Godbert, S., Van De Braak, T. G. H., Dunn, I. C. & Review What Are the Challenges Facing the Table Egg Industry in the next Decades and What Can Be Done to Address Them? *Animal 15*, 100282. (2021). https://doi.org/10.1016/j.animal.2021.100282
2. Leenstra, F., Ten Napel, J., Visscher, J. & Van Sambeek, F. Layer breeding programmes in changing production environments: A historic perspective. *World's Poult. Sci. J.* **72** (1), 21–36. https://doi.org/10.1017/S0043933915002743 (2016).
3. Sakomura, N. K., Reis, M. D. P., Ferreira, N. T. & Gous, R. M. Modeling egg production as a means of optimizing dietary nutrient contents for laying hens. *Anim. Front.* **9** (2), 45–51. https://doi.org/10.1093/af/vfz010 (2019).
4. Jia, N. et al. A review of key techniques for in Ovo sexing of chicken eggs. *Agriculture* **13** (3), 677. https://doi.org/10.3390/agriculture13030677 (2023).
5. Steiner, G. et al. Gender determination of fertilized unincubated chicken eggs by infrared spectroscopic imaging. *Anal. Bioanal Chem.* **400** (9), 2775–2782. https://doi.org/10.1007/s00216-011-4941-3 (2011).
6. Galli, R. et al. In Ovo sexing of domestic chicken eggs by Raman spectroscopy. *Anal. Chem.* **88** (17), 8657–8663. https://doi.org/10.1021/acs.analchem.6b01868 (2016).
7. Corion, M. et al. Trends in in Ovo sexing technologies: insights and interpretation from papers and patents. *J. Anim. Sci. Biotechnol.* **14** (1), 102. https://doi.org/10.1186/s40104-023-00898-1 (2023).
8. Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. A DNA test to sex most birds. *Mol. Ecol.* **7** (8), 1071–1075. https://doi.org/10.1046/j.1365-294x.1998.00389.x (1998).
9. He, L. et al. Simple, sensitive and robust chicken specific sexing assays, compliant with large scale analysis. *PLoS ONE.* **14** (3), e0213033. https://doi.org/10.1371/journal.pone.0213033 (2019).
10. Brubaker, J. L. et al. Direct Real-time PCR method for sex determination in multiple avian species. *Mol. Ecol. Resour.* **11** (2), 415–417. https://doi.org/10.1111/j.1755-0998.2010.02951.x (2011).
11. Chang, H. W. et al. High-Throughput avian molecular sexing by SYBR Green-Based Real-Time PCR combined with melting curve analysis. *BMC Biotechnol.* **8** (1), 12. https://doi.org/10.1186/1472-6750-8-12 (2008).
12. Chen, C. C. et al. High-Throughput sex identification by melting curve analysis in Blue-Breasted quail and chicken. *Theriogenology* **77** (9), 1951–1958. https://doi.org/10.1016/j.theriogenology.2011.12.004 (2012).
13. Clinton, M., Haines, L., Belloir, B. & McBride, D. Sexing chick embryos: A rapid and simple protocol. *Br. Poult. Sci.* **42** (1), 134–138. https://doi.org/10.1080/713655025 (2001).
14. Cordeiro, C. D. et al. Accurate, and Cost-Effective poultry sex genotyping using Real-Time polymerase chain reaction. *Front. Vet. Sci.* **10**, 1196755. https://doi.org/10.3389/fvets.2023.1196755 (2023).
15. Hou, B. et al. Development of a sensitive and specific multiplex PCR method for the simultaneous detection of chicken, Duck and Goose DNA in meat products. *Meat Sci.* **101**, 90–94. https://doi.org/10.1016/j.meatsci.2014.11.007 (2015).

16. Clinton, M. et al. Real-Time sexing of chicken embryos and compatibility with in Ovo protocols. *Sex. Dev.* **10** (4), 210–216. https://doi.org/10.1159/000448502 (2016).
17. Notomi, T. & Loop-Mediated Isothermal amplification of DNA. *Nucleic Acids Res.* **28** (12), 63e–663. https://doi.org/10.1093/nar/28.12.e63 (2000).
18. Crego-Vicente, B., Del Olmo, M. D., Muro, A., Fernández-Soto, P. & Multiplexing LAMP Assays: A Methodological Review and Diagnostic Application. *IJMS 25* (12), 6374. (2024). https://doi.org/10.3390/ijms25126374
19. Moehling, T. J., Choi, G., Dugan, L. C., Salit, M. & Meagher, R. J. LAMP diagnostics at the Point-of-Care: emerging trends and perspectives for the developer community. *Expert Rev. Mol. Diagn.* **21** (1), 43–61. https://doi.org/10.1080/14737159.2021.1873769 (2021).
20. Soroka, M., Wasowicz, B. & Rymaszewska, A. Loop-Mediated isothermal amplification (LAMP): the better sibling of PCR? *Cells* **10** (8), 1931. https://doi.org/10.3390/cells10081931 (2021).
21. Sharma, S., Singh, J., Sen, A. & Anvikar, A. Multiplex loop mediated isothermal amplification (m-LAMP) as a point of care technique for diagnosis of malaria. *J. Vector Borne Dis.* **59** (1), 29. https://doi.org/10.4103/0972-9062.331409 (2022).
22. Kumar, S. et al. Advanced lyophilised loop mediated isothermal amplification (L-LAMP) based point of care technique for the detection of dengue virus. *J. Virol. Methods*. **293**, 114168. https://doi.org/10.1016/j.jviromet.2021.114168 (2021).
23. Schneider, F. S. et al. Performances of rapid and connected salivary RT-LAMP diagnostic test for SARS-CoV-2 infection in ambulatory screening. *Sci. Rep.* **12** (1), 2843. https://doi.org/10.1038/s41598-022-04826-7 (2022).
24. Choi, G., Moehling, T. J. & Meagher, R. J. Advances in RT-LAMP for COVID-19 testing and diagnosis. *Expert Rev. Mol. Diagn.* **23** (1), 9–28. https://doi.org/10.1080/14737159.2023.2169071 (2023).
25. Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA detection using recombination proteins. *PLoS Biol.* **4** (7), e204. https://doi.org/10.1371/journal.pbio.0040204 (2006).
26. Li, J., Macdonald, J., Von Stetten, F. & Review A comprehensive summary of a decade development of the recombinase polymerase amplification. *Analyst* **144** (1), 31–67. https://doi.org/10.1039/C8AN01621F (2019).
27. Papadakis, G. et al. Portable Real-Time colorimetric LAMP-Device for rapid quantitative detection of nucleic acids in crude samples. *Sci. Rep.* **12** (1), 3775. https://doi.org/10.1038/s41598-022-06632-7 (2022).
28. Zhang, T. et al. Rapid and simple detection of glaesserella parasuis in synovial fluid by recombinase polymerase amplification and lateral flow strip. *BMC Vet. Res.* **15** (1), 294. https://doi.org/10.1186/s12917-019-2039-x (2019).
29. Li, X. et al. Recombinase polymerase amplification coupled with a photosensitization colorimetric assay for fast *Salmonella* spp. Testing. *Anal. Chem.* **93** (16), 6559–6566. https://doi.org/10.1021/acs.analchem.1c00791 (2021).
30. Fridolfsson, A. K. & Ellegren, H. A. Simple and universal method for molecular sexing of Non-Ratite birds. *J. Avian Biol.* **30** (1), 116. https://doi.org/10.2307/3677252 (1999).

Acknowledgements

The authors thank Louis Perrault and Saint Marcellin hatchery for the technical support during the study. We thank the France Agrimer Institution for funding project. The funders had no role in designing or directing the study and editing the manuscript.

Author contributions

Conceptualization: TNN.V, J.E and F.M. Methodology: TNN.V. Investigation: M.VDH, N.L'H, J.HC, PGA.M, T.M, T.G, J.H, J.E and TNN.V. Data analysis and visualization: M.VDH. Data curation: M.VDH and TNN.V. Validation: M.VDH and TNN.V. Funding acquisition: F.M. Writing – original draft: TNN.V. Writing & editing: M.VDH, J.E and TNN.V. Reviewing the final manuscript: M.VDH, N.L'H, J.HC, PGA.M, T.M, T.G, J.H, J.E, FM and TNN.V.

Funding

The study was a part of SOO project funded by the France Agrimer Institution.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The Institutional Animal Care and Use Committee (IACUC), an Association of New England Medical Center and Tufts, as well as the National Institutes of Health, USA, mandated that a chick embryo that has not reached the 14th day of its gestation period would not experience pain and can therefore be used for experimentation without any ethical restrictions or prior protocol approval, simplifying the planning process.
The European Directive 2010/63/EU transposed into French law, which explicitly excludes early embryonic stages (< 14 days for chickens) from the scope of authorization procedures. The Decree No. 2022 – 137 of February 6, 2022, which sets the legal threshold at D15 for in ovo sexing in the poultry sector, implicitly confirming the absence of legal protection before this stage.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-13013-3.

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