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Real-Time Sexing of Chicken Embryos and Compatibility with in ovo Protocols

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Key Words
Chicken · Development · Embryo · in ovo · Sexing

Abstract
The chicken embryo is an established model system for studying early vertebrate development. One of the major advantages of this model is the facility to perform manipulations in ovo and then continue incubation and observe the effects on embryonic development. However, in common with other vertebrate models, there is a tendency to disregard the sex of the experimental chicken embryos, and this can lead to erroneous conclusions, a lack of reproducibility, and wasted efforts. That this neglect is untenable is emphasised by the recent demonstration that avian cells and tissues have an inherent sex identity and that male and female tissues respond differently to the same stimulus. These sexually dimorphic characteristics dictate that analyses and manipulations involving chicken embryos should always be performed using tissues/embryos of known sex. Current sexing protocols are unsuitable in many instances because of the time constraints imposed by most in ovo procedures. To address this lack, we have developed a real-time chicken sexing assay that is compatible with in ovo manipulations, reduces the number of embryos required, and conserves resources.

The chicken embryo is a long-established model organism that has enabled major advances in all areas of developmental biology. This model has contributed to defining basic principles such as induction and competence, establishing general mechanisms such as asymmetry and patterning, and elucidating the genetic regulation underlying the specification of individual tissues/organs (e.g., neural development, limb development) [reviewed in Levin et al., 1995; Stern, 2004; Davey and Tickle, 2007]. Benefits of this model include: (a) a shelled egg that is independent of the hen, allowing easy access for observation and experimental manipulation; (b) the ability to synchronise the incubation of large numbers and isolate embryos at precise stages of development; (c) the comparatively large size; (d) a planar development that shows significant similarities to that of the early human embryo; and (e) the fact that fertilised eggs are both inexpensive and readily available. The different stages of chicken development have been documented in great detail by Hamburger and Hamilton [Hamburger and Hamilton, 1951] amongst others, and a wide variety of experimental procedures have been developed for this model. Manipu-

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lations can be performed at different developmental stages via a small window cut in the egg shell, and the egg can then be sealed and re-incubated, allowing further embryonic development – an approach that is extremely difficult with placental vertebrates.

In common with other vertebrate model systems, the majority of chicken studies fail to consider the sex of embryos. However, research on mammalian systems has shown that this oversight is no longer supportable and that both sexes should be considered in an experimental design: the bias towards including only male animals or male cells results in a failure to identify sex differences relevant to downstream clinical studies and may contribute to a lack of reproducibility in many studies [Clayton and Collins, 2014]. As regards non-mammalian vertebrates, we have recently shown that chicken somatic cells possess an inherent sex identity and that male and female cells and tissues respond differently to extracellular stimulation [Zhao et al., 2010]. Even non-reproductive tissues exhibit hormone-independent sexually dimorphic characteristics [Clinton et al., 2012; Maekawa et al., 2013; Garcia-Morales et al., 2015]. As a consequence, the use of unsexed embryos in chicken studies could confound experimental results: it will certainly lead to an increase in variation in the parameters under study and could produce completely misleading conclusions. For instance, transcriptomic comparisons on pools of tissue from unsexed embryos will identify gene expression differences that are simply due to differences in the male:female composition of the samples, and that are unrelated to the primary focus of the analysis. Similarly, in grafting studies, heterologous transplants are unlikely to behave in exactly the same way as homologous transplants, and male and female embryos may even respond differently to expression from electroporated constructs or to the effects of implanted beads. For this reason, such studies should always be performed on embryos of known sex, and ideally, on embryos of both sexes. A reliable molecular sexing method is required as the majority of tissue manipulations/collections are performed when male and female embryos are morphologically indistinguishable. There are currently a number of methods available, but these are laborious and time-consuming and incompatible with studies involving live embryos [Clinton, 1994; Griffiths et al., 1998; Cortes et al., 1999; Fridolfsson and Ellegren, 1999; Clinton et al., 2001; Chang et al., 2008]. To address this requirement, we have developed a simple and robust assay that allows the rapid identification of the sex of individual embryos using a small quantity of crude material. With this procedure, sex can be determined in 5–15 min using either tissue fragments, small volumes of whole blood, or a small number of isolated cells.

Materials and Methods

Biological Materials

Fertile eggs from ISA Brown hens were incubated at 38 ± 0.5°C for the desired time.

For routine tissue collections, embryos were decapitated and the relevant tissue was dissected and snap frozen. A fragment (~50 mg) of the remaining embryo was excised and either processed immediately or stored at –20°C. For analysis, water (100 µl) was added to the tissue and the sample was heated at 95°C for 5 min. Without further processing, 7.5 µl was transferred to a 96-well plate for assaying.

For procedures involving live embryos, a small volume (~0.5 µl) of blood was collected, diluted to 10 µl with water, and heated at 95°C for 5 min. After cooling, 7.5 µl was used in the Hologic Invader® sexing assay. Depending on the stage of development involved, blood was collected from either a chorio-allantoic membrane vessel, the vitelline vein, or the heart.

Blood was collected from adult birds of known sex into PBS containing EDTA to a final concentration of 5 mM and a series of 2-fold dilutions prepared in PBS/EDTA. A small volume of diluted blood (equivalent to 0.063–2.0 µl of whole blood) was adjusted to 7.5 µl with water, heated to 95°C, and used in the Hologic Invader® sexing assay.

Blood collected from 5 male and 5 female adult birds was used to prepare genomic DNA by standard procedures [Sambrook, 1989].

Five independent cultures of male and female chicken embryo fibroblasts (CEF) were prepared [Hernandez and Brown, 2010] from embryos at day 16 of development. Embryo sex was determined by visual examination of the gonads.

Hologic Invader® Sexing Assay

The Invader® sexing assay reagents are proprietary materials owned and produced by Hologic Inc (10210 Genetic Center Drive, San Diego, Calif., 92121, USA) (http://www.hologic.com/). The Hologic Invader® sexing assay comprises a 'W-rpt/CR2 probe mixture' (catalogue number AGBIOTG-XS-006) and a Reagent Core Kit' (catalogue number 91–219). The W-repeat probe mixture comprises a probe and oligo targeting nucleotides 1–200 of the chicken W chromosome-specific repetitive DNA (Xho1 family) [genebank: X06548.1] and a FRET cassette (FAM: excitation 494 nm, emission 520 nm), and a probe and oligo targeting nucleotides 4021–4558 of chicken retrotransposon CR1 consensus sequence [genebank: U88211.1] and FRET cassette (Redmond RED: excitation 575 nm, emission 602 nm). The Reagent Core kit contains the Cleavase enzyme.

Probes, FRET cassettes, and enzyme are combined in a 2× Master mix. The samples to be assayed are diluted in water, heated at 95°C for 5 min, and then combined with an equal volume of 2× Master mix. Samples are then incubated at 63°C in a Stratagene MxPro 3000 thermal cycler and fluorescence monitored (using both FAM and ROX filters) at 10 s and at 1-min intervals thereafter.

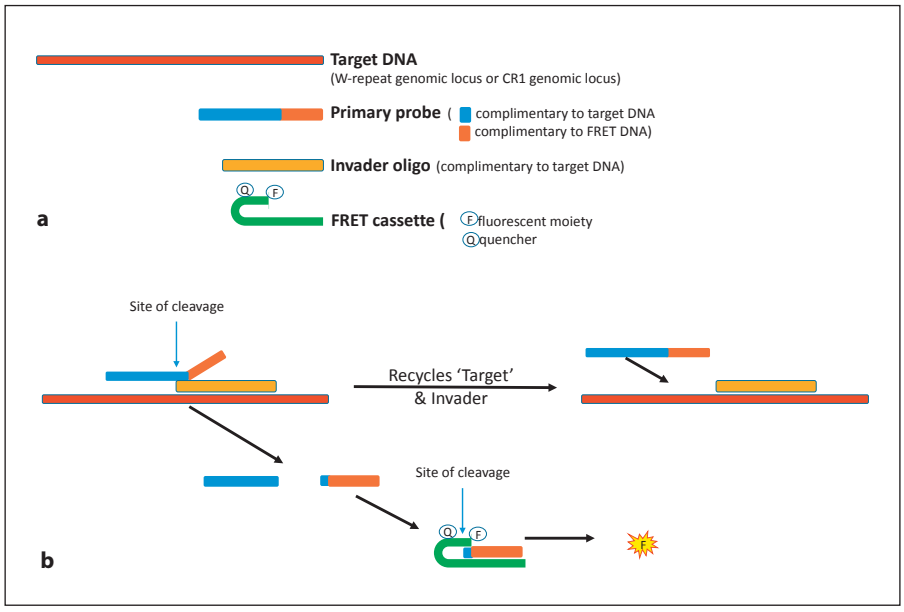

Fig. 1. Schematic illustrating Invader sexing assay. **a** The nucleic acid components of the sexing assay. **b** Generation of the fluorescence signal. The overlapping structure is recognised by a structure-specific endonuclease that cleaves the primary probe and releases the 5′-oligo. The target-specific 5′-oligo acts as an 'invader' probe on the FRET cassette and is cleaved by the endonuclease, generating the fluorescence signal. The target and primary probe structure, and the 5′-Flap oligo are recycled.

Statistics

A minimum of 5 male and female biological replicates were used at all stages of assay validation. Signal values for male and female samples were compared by Student t test.

Results

Basic Assay Principle

The sexing assay is based on Hologic Invader® technology (http://invaderchemistry.com/), an isothermal 'PCR-free' approach that exploits a thermostable structure-specific archaebacterial flap endonuclease (FEN) that cleaves nucleic acid molecules at specific sites based on structure rather than sequence. When used in conjunction with structure-forming probes for known sequences, this 'Cleavase' enzyme cuts in a structure- and target sequence-specific manner [de Arruda et al., 2002].

For each target sequence, 2 oligonucleotide probes (a primary probe and an Invader® probe) hybridise in tandem to the target DNA to form a specific overlapping structure (fig. 1). The 5′-end of the primary probe contains a 5′-Flap that is non-complementary to the target DNA and so is unable to hybridise to the target sequence. The 3′-end of the bound Invader® probe overlaps the 5′-end of the target complementary portion of the primary probe. The Cleavase® enzyme recognises the overlapping structure and cleaves the primary probe releasing the 5′-Flap as a target specific product. The target specific 5′-Flap oligos are then involved in a secondary reaction where they act as Invader® probes on a fluorescent resonance energy transfer (FRET) cassette forming an overlapping structure that is also recognised by the Cleavase® enzyme. When the FRET cassette is cleaved, a fluorophore is released from a quencher on the FRET, allowing for the generation of a target-specific detectable fluorescence signal.

The primary probe has a melting temperature close to the reaction temperature (63°C) and the assay cycles continuously: following cleavage of the primary probe, the remaining portion of the original probe is released and replaced by an uncleaved probe which, in turn, is cleaved and released. This allows for multiple rounds of primary probe cleavage for each target and amplification of the number of released 5′-flaps. As with the initial reaction, the 5′-flap and FRET cassette cycle and amplify the fluorescence signal. As probes are present in excess, multiple rounds of primary probe cleavage occur per DNA target and multiple fluorescence signal fluorophores are generated per DNA target released. The assay uses 2 different discriminatory primary probes and 2 different FRET cassettes, each with a spectrally distinct fluorophore. Both reactions run together, allowing the simultaneous detection of 2 DNA target sequences in a single well.

The 2 target sequences utilised in the chicken sexing assay are the W chromosome Xho1 repeat sequence [Tone et al., 1982; Kodama et al., 1987; Saitoh et al., 1991] that is specific to the female genome and the CR1-repeat sequence [Vandergon and Reitman, 1994; Coullin et al.,

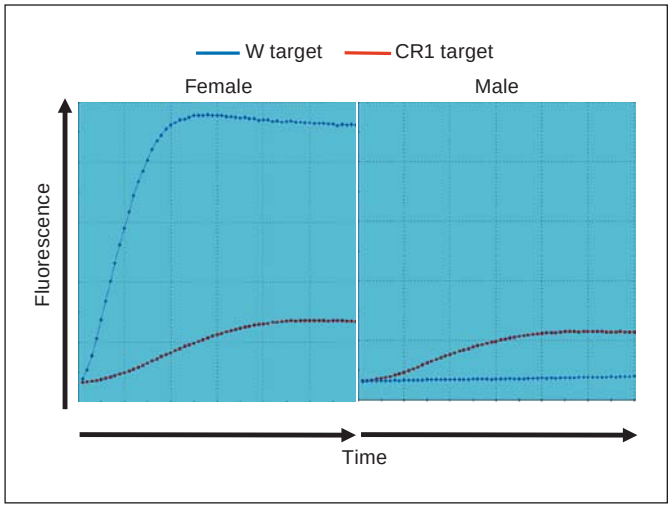

Fig. 2. Typical fluorescence profiles. Screenshots from a plate reader showing fluorescence signals generated by female and male samples over time. The signal accumulating from the W chromosome female-specific probe (W target) is shown in blue and the signal from male and female common repeat sequence (CR1 target) is shown in red.

2005] that is common to both males and females. Fluorescence signal from the cleavage of the common CR1 sequence ensures the presence of DNA, whereas generation of the female-specific W-repeat signal determines the sex of the sample. Figure 2 shows the typical fluorescence profiles generated in real-time for both the W-repeat sequence and the CR1-repeat sequence.

Accuracy

For routine sexing of embryos of different developmental stages, we utilise a standard tissue fragment appropriate for that particular stage e.g., a toe from a day 6.5 embryo. This tissue is transferred to a tube containing 100 µl of water and heated at 95°C for 5 min. Without further processing, 7.5 µl is then transferred to a 96-well plate. Reagent mix (7.5 µl) is added and the plate incubated at 63°C, and the fluorescence is measured at 520 nm (FAM) and 602 nm (ROX) after 10 s of incubation and at 1 min intervals thereafter. Figure 3 depicts a screenshot showing the typical fluorescence profiles obtained from a 96-well plate containing tissue lysate from 76 embryos from 2 separate collections. 'Control' samples comprised female DNA, male DNA, and a water 'blank'. If only a small number of samples are to be analysed, sex can be assigned to individual samples simply by visual examination of the fluorescence profiles in real time. If desired, for larger sample numbers, fluorescence measurements can be exported in Microsoft Excel format at selected time points and a simple formula used to automatically assign the sex of individual samples. Briefly, values obtained at the selected time point (e.g., 15 min) for the W and CR1 sequences are corrected for background readings at 10 s (fluorescence at t_{15m} – fluorescence at t_{10s}). The W-repeat signal is then expressed relative to the CR1 signal ([FAM t_{15m}–t_{10s}] – [ROX t_{15m}–t_{10s}]), and samples with an overall positive value are automatically designated as female and samples with a negative value are automatically designated as male (online suppl. table S1 details the fluorescence measurements and calculations for samples in figure 3 and online suppl. fig. S1 illustrates fluorescence profiles and derived overall fluorescence values at different time points for samples A1–A12; for all online suppl. material, see www.karger.com/doi/10.1159/000448502). Even with crude tissue lysate, 5–10 min of incubation is clearly sufficient to discriminate between male and female samples. In instances where only a very limited quantity of material is available, the incubation time can be extended and the accumulation of fluorescence signal monitored visually. During assay development, each sample analysed (n >500) was also sexed using an established molecular sexing assay. In addition, the Hologic Invader® assay was used in a blind test to assess blood samples from 1,000 adult birds (data not shown). In all instances, the Hologic Invader® assay results agreed with either the known sex of the adult birds or the sex of embryos as determined by an established protocol.

Sensitivity

Assay sensitivity was established using different quantities of purified male and female genomic DNA. The fluorescence profiles and the overall signal generated by different dilutions of male and female DNA, over 20 min, are illustrated in online suppl. figure 2 and show that female and male samples can be discriminated using as little as 1 ng of genomic DNA. Figure 4a shows the W-specific fluorescence signal generated by 32 ng of male and female DNA at different time points. The female samples show a steady increase in fluorescence with a maximum signal attained between 17–20 min, while male samples show no increase above the background. In contrast, there is no difference between the CR1-repeat signal generated by male and female DNA (fig. 4b), which steadily accumulates in both male and female samples throughout the time period studied. When the W-repeat signal is 'normalised' against the CR1 signal ([FAM t_x–t_{10s}]–[ROX t_x–t_{10s}]) the overall derived value for a male DNA sample is negative

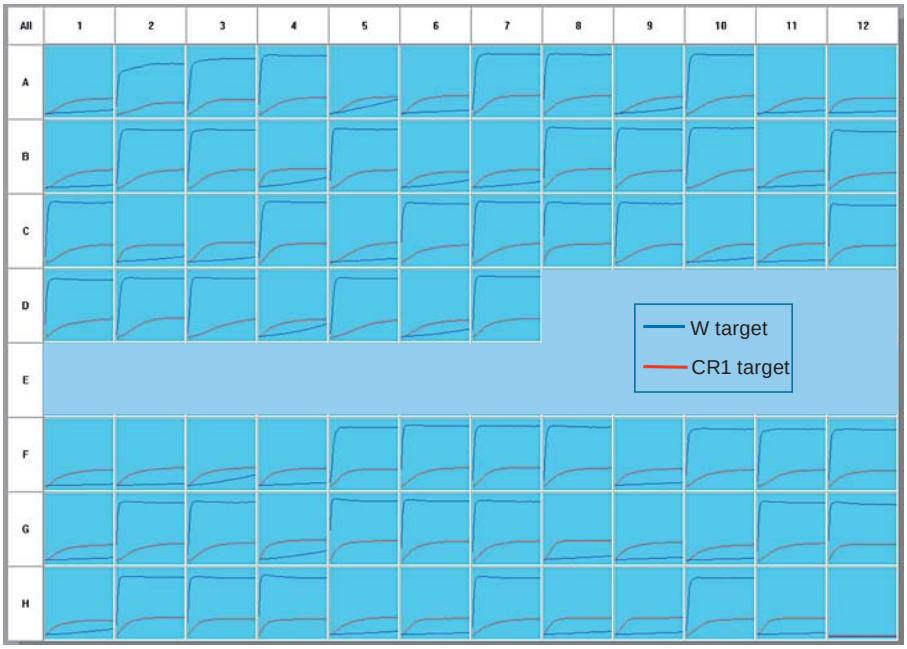

Fig. 3. Sexing of crude tissue lysates. Screenshot from a plate reader of a 96-well plate showing fluorescence profiles generated by lysates from 76 embryos of unknown sex and 3 control wells (H10: female DNA, H11: male DNA, H12: water). The sex of individual embryos is obvious from visual examination of the fluorescence profiles.

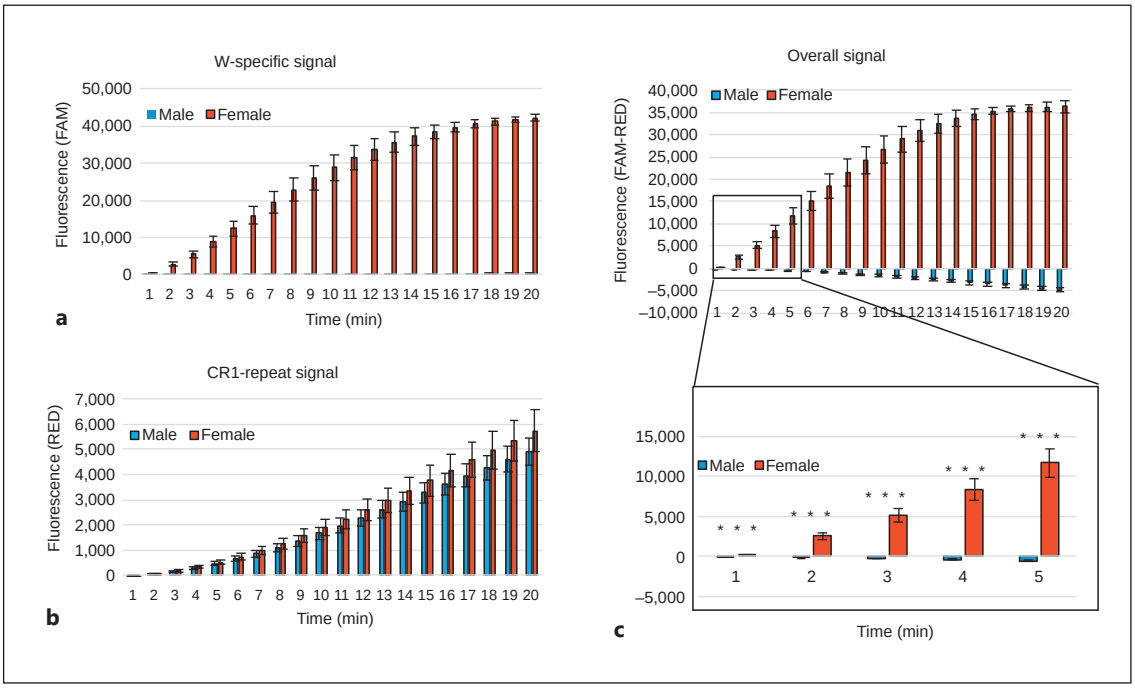

Fig. 4. Fluorescence profiles from female and male DNA. **a** Fluorescence signal generated by the W-repeat reaction from 32 ng of male and female DNA ($FAMt_x$–t_{10s}) over time. **b** Fluorescence signal generated by the CR1-repeat reaction from 32 ng of male and female DNA ($ROXt_x$–t_{10s}) over time. **c** Overall fluorescence signal derived from female and male W-repeat and CR1-repeat reactions over time ([$FAMt_x$–t_{10s}]-[–OXt_x–t_{10s}]). **Inset** shows enlarged image of 1–5 min time points (n = 5; *** $p < 0.005$; error bars represent 1 SD).

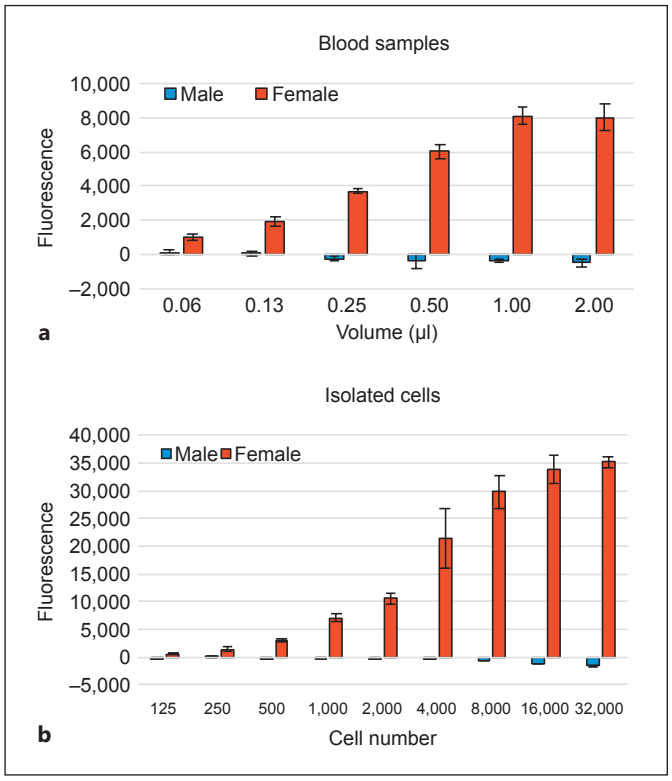

Fig. 5. Sexing of materials for procedures involving live embryos. **a** Signals (at 15 min) generated by different volumes of whole blood. The overall fluorescence signal for individual samples was derived ($[FAMt_{15}–t_{10s}]–[ROXt_{15}–t_{10s}]$) and plotted against the blood volume. Male and female samples could be discriminated using as little as 0.125 µl of blood ($n = 5$; $p < 0.005$). **b** Signals (at 15 min) generated by different numbers of cells. The overall fluorescence signal for individual samples was derived ($[FAMt_{15}–t_{10s}]–[ROXt_{15}–t_{10s}]$) and plotted against the cell number. Male and female samples could be discriminated using as few as 250 cells ($n = 5$; $p < 0.005$; error bars represent 1 SD).

and decreases with incubation time, while the derived value of a female DNA sample is positive and increases with incubation time (fig. 4c). The difference between the signals generated by male and female samples is statistically significant as early as the 1 min time point ($p < 0.005$).

Compatibility with in ovo Protocols

To explore the utility of using the Hologic Invader® sexing assay for in ovo experiments, we evaluated its performance on small volumes of whole blood. Blood was collected from 5 male and 5 female embryos and used to generate a series of 2-fold dilutions directly in a 96-well plate. Each sample was adjusted to a total volume of 7.5 µl and heated to 95°C and incubated at 63°C following the addition of the assay reagents. The signals generated after 15 min incubation are shown in figure 5a, and samples can clearly be identified as either male or female from as little as 0.125 µl of whole blood.

We also utilised the assay to sex embryos prior to the formation of the circulatory system (data not shown). These embryos were 'donors' used to provide material for transplantation studies: blastoderm cells from newly laid eggs and lateral plate mesoderm from H&H Stage 12 embryos. In both instances, we were able to use small fragments of unused tissue to identify the sex of the donor embryos, while material was being prepared for transplantation e.g., washing yolk from blastoderm cells. This enabled us to pool blastoderm cells of the same sex prior to injection and to avoid the generation of unwanted heterologous transplants. To determine the minimum number of cells required for such studies, we tested the sexing assay on isolated CEFs. Five male and 5 female CEF cultures were harvested, counted, and used to generate a series of 2-fold dilutions. The signals generated by different numbers of cells after 15 min of incubation are shown in figure 5b. The sex of individual samples can be determined using as few as 250 cells.

Discussion

The use of the chicken embryo as a model system has led to major advances in our understanding of all aspects of vertebrate development. A significant advantage of the chicken embryo model is the ability to manipulate the embryo at different stages and then to continue incubation and observe the effects of that treatment on embryonic development. Until recently, the necessity to identify the sex of manipulated embryos was considered to be restricted to studies involving reproductive tissues. However, we have now established that chicken somatic cells/tissues possess either an inherent male or an inherent female identity, and this has implications for all chicken embryo studies. This cell-autonomous sex identity (CASI) was demonstrated by the fact that male cells transplanted into female embryos continue to differentiate and behave/respond in a male-specific manner, and vice versa. For example, male cells in female gonads express AMH, while female cells in male gonads express aromatase [Zhao et al., 2010]. While the developmental consequences of CASI will be more pronounced in tissues that are clearly sexually dimorphic, such as the gonads, as a result of CASI there will be structural and molecular male:female differences in all tissues [Maekawa et al., 2013]. As a consequence, ideally, all chicken embryo studies should use

material derived from embryos of known sex. While there are a number of molecular sexing assays available that are adequate for routine tissue collection and downstream analysis studies, these are somewhat laborious and time-consuming and are not suitable for studies involving in ovo manipulation of live embryos. To address this issue, we have developed a novel sexing procedure based on Hologic Invader® technology. This assay is accurate, sensitive and rapid, and can distinguish the sex of individual animals from as little as 1 ng of DNA or 125 nl of whole blood, or as few as 250 cells. The assay is less prone to cross-contamination than current protocols, has minimal equipment requirements (heat source and plate reader), and template purification is unnecessary – being routinely applied to heat-lysed cells or tissues.

In summary, while the chicken embryo is an excellent vertebrate model, many of the protocols devised for this system require a high degree of expertise and involve a considerable investment of resources. Due to sexual dimorphisms, unintended and unwanted heterologous transplants/treatments represent a significant waste. The ability to identify the sex of donor embryos prior to transplantation or recipient embryos prior to electroporation/implantation will result in a considerable saving in time and effort.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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