Limitation of standard pseudoisochromatic plates in identifying colour vision deficiencies when compared with genetic testing

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ABSTRACT.

Purpose: The Ishihara pseudoisochromatic (PIC) plate test is the most used test for identifying red-green colour-deficient individuals, but it is not known how the Ishihara results compare with that of genetics testing. Here, the outcome of genotype analysis of $OPN1LW$ and $OPN1MW$ was compared with that of the Ishihara (24-plate ed., 1964) and the Hardy-Rand-Rittler (4th ed. 2002) PIC plate tests.

Methods: Healthy participants with normal habitual visual acuity ($n = 454$, 16–24 years; 193 males; logMAR $\leq 0.00$) gave saliva samples for opsin gene analysis and performed the two PIC plate tests as part of a cross-sectional study. The criteria for failing the PIC tests were according to manufacturers’ instructions. DNA was extracted and used in genotyping assays of $OPN1LW$ and $OPN1MW$ genes from each participant using the Agena MassArray genotyping system.

Results: Ten male (5.2%) and 3 (1.1%) female participants were identified as red-green colour deficient based on PIC tests alone. The combination of MassArray and PIC test results identified 10.4% of male and 0.8% of female participants to be colour deficient (males: 0.5% protan and 9.9% deutan; females: 0.8% deutan). Hardy–Weinberg calculations based on male frequencies from combining the MassArray and the PIC test results gave female frequency estimates of colour deficiency and carriers closely matching measured frequencies.

Conclusions: MassArray identified twice as many colour-deficient males as identified from PIC tests alone. Combining results from MassArray and the PIC tests proves to be more reliable than any single test at correctly identifying red-green colour-deficient individuals and carriers.

Key words: colour vision deficiency – colour vision testing – genetic testing – Hardy-Rand-Rittler test – Ishihara test – opsin genes

Introduction

Congenital colour vision deficiencies are caused by inherited photopigment abnormalities (Nathans et al. 1986a; Nathans et al. 1986b) and impair the affected individual’s ability to discriminate colours. The most common form is red-green colour vision deficiencies, which is inherited via the X-chromosome. Red-green colour vision deficiencies are reported to affect 8–10% of male and about 0.5% of female Caucasians with at least 15% of females being carriers of the deficiency when Rayleigh anomaloscopy has been employed for testing (Waaler 1927; Gegenfurtner & Sharpe 1999). The extensive use of colour coding in daily life, colour light signals in the transport industries and use of colour for teaching and learning in all levels of education have been shown to limit an affected individual’s possibilities for reaching their potential and/or hinder entrance into certain occupations (Birch 1993). It is, therefore, considered favourable both for the affected individual and the society to identify those who are affected. The most common way for identifying affected individuals is by administering a pseudoisochromatic (PIC) plate test. Pseudoisochromatic (PIC) tests for screening of colour vision deficiencies have been around for more than a 100 years. The Ishihara colour vision test was first published in 1906 by Dr. Shinobu Ishihara and the Hardy-Rand-Rittler PIC test was first
published in 1955 by American Optical (Dain 2004).

The Ishihara test is probably the most used PIC test in the world. It can be used to test for red-green colour vision deficiencies only, and each plate consists of a number or a path embedded in a random pattern of coloured dots. Sensitivity and specificity for three errors has been reported to be 98.4% (Birch 2010) and 94.1% (Birch 1997), respectively, when comparing with Rayleigh anomaloscopy. The accuracy of the test is reported to be better when administered by an experienced practitioner, but it cannot be used to predict the type and degree of colour-deficient individuals (Neitz & Neitz 2000; Dain 2004; Miyahara 2008). Despite these limitations, it is often the only test used when screening children or for entrance into different occupations, for example in the transport industry (International Commission on Illumination 2001).

The current version of the Hardy-Rand-Rittler PIC test (HRR; published in 2002, fourth edition, Richmond Products, Albuquerque, NM, USA) can be used to test for both red-green and tritan colour vision deficiencies. Each plate consists of either one- or two-coloured symbols embedded in a random pattern of grey dots. The test includes both screening and diagnostic plates for predicting type and grading the severity of colour vision deficiencies. Sensitivity for 2 errors has been reported to be 92.8% (when only the four screening plates have been employed) (Birch 2010), but 100% when completing the test (Bailey et al. 2004), when comparing with Rayleigh anomaloscopy. The predicted severity has also been reported to correspond closely with that determined by the anomaloscope (Bailey et al. 2004), except that variation in pre-retinal filtering and polymorphism in the L and M cone photopigments may cause some dichromats to be classified as moderate severity by the HRR (Davidoff et al. 2016). Rayleigh anomaloscopy is considered the gold standard for sensitivity in detecting red–green colour vision deficiencies (Waaler 1927), but there is as of yet no study assessing the sensitivity and specificity of Rayleigh anomaloscopy compared with genotyping. It is known that some individuals with opsin genes conferring red-green colour vision deficiency with a small spectral separation between their photopigments, set a Rayleigh match that is better than expected (Shevell et al. 1998; Barbur et al. 2008). Furthermore, there are several reports on individuals who make no errors on neither the Ishihara nor the HRR tests, but who set a Rayleigh match that is anomalous (e.g. Ref. (Dees et al. 2015)).

The knowledge that it is difficult to identify everyone who has a red-green colour vision deficiency with PIC tests alone (Birch 2010) has signified the need for including genetic testing to identify colour vision deficiency in a more reliable way. Detailed genetic analysis of cone opsin genes and inheritance of colour vision deficiencies was first reported by Nathans et al. (1986a) and Nathans et al. (1986b). Later work has been reviewed by Deeb (2005) and Neitz and Neitz (2011). Genotyping of cone opsin genes using the Agena MassArray technology was developed by Neitz and colleagues and has been reported to reliably classify red-green colour vision deficiencies (Davidoff et al. 2016). They compared OPN1LW/OPN1MW genotype data with a new minimalistic paper and pencil test and the HRR PIC test. To date, it is not known how the outcome of the Ishihara test compares with MassArray genotypes for identifying red-green colour-deficient males, nor for identifying red-green colour-deficient females and carriers. The aim in this study was, therefore, to compare and combine results from MassArray analysis with that of the Ishihara (24 pl. ed.) and the HRR (4th ed.) PIC tests and assess the PIC tests’ ability to correctly identify red-green colour-deficient and colour normal individuals.

Methods

Participants

Male and female participants with normal habitual visual acuity ($n = 454$, 16–24 years; 193 males; 408 Caucasians; logMAR ≤0.00) were asked to give saliva samples for opsin gene analysis and perform colour vision tests as part of a cross-sectional study on refractive errors (Hagen et al. 2018). The participants comprised a representative sample of the Norwegian population for the given age group (Hagen et al. 2018).

Informed consent was obtained from all participants after explanation of possible consequences of the study and prior to the experiments. The research was approved by the Regional Committee for Medical Research Ethics for the South-East Norway Regional Health Authority and by an Institutional Review Board at the University of Washington. All research was conducted in accordance with the principles embodied in the Declaration of Helsinki.

MassArray genotyping

All participants gave saliva samples using the Oragene 500 collection kit (DNA Genotek, Ottawa, ON, Canada), and DNA was extracted in adherence to the manufacturer’s protocol. The DNA was subsequently used in genotyping assays to estimate the relative number of OPNILW and OPNIMMW genes on the X-chromosome of each participant. Five different single nucleotide polymorphisms (SNPs) were genotyped using the Agena MassArray system (Agena Bioscience Inc., San Diego, CA, USA).

The most critical SNPs for this study were the SNP in exon 5 codon 309 that distinguishes OPNILW from OPNIMMW genes in the majority of people, and the SNP in the promoter region at the +1 position that, in most people, distinguishes the first opsin gene from all downstream opsin genes in the X-chromosome opsin gene array. The other three SNPs examined were in exons 2, 3 and 4, with one SNP per exon. A detailed description of the MassArray genotyping assay for colour vision has been described previously (Davidoff et al. 2016). DNA from each participant was also used in the polymerase chain reaction (PCR), to selectively amplify a region from intron 1 to exon 5 of the OPNILW and OPNIMMW genes using procedures described in detail previously (Neitz et al. 2004). The genetic analyses were performed in the Neitz Lab at University of Washington, Seattle.

Colour vision testing

Colour vision testing was performed with the Ishihara (24-plate ed., 1964) and the Hardy-Rand-Rittler (4th ed. 2002) PIC plates under 781 (±67) lux.
The criteria chosen for failing the Ishihara and HRR tests were according to manufacturers’ instructions that is three or more typical errors on the Ishihara and two or more errors on the screening plates 7–10 made upon retesting for the HRR. For further analyses, a combined criterion was employed for classifying an individual as red-green colour deficient based on the two PIC tests. That was, the total sum of errors for both tests were set to five errors or more. For example, an individual who made two errors on the HRR screening plates upon retesting plus one error on the diagnostic plates and two errors on the Ishihara were classified as red-green colour deficient. Five of the males who made errors on PIC tests that indicated red-green colour deficiency and a random sample of 22 males and 24 females were invited to perform Rayleigh anomaloscopy with the dominant eye (HMC Oculus Anomaloscope MR, Typ 47 700, Oculus Optikgeräte GmbH, Wetzlar, Germany; method described elsewhere (Dees et al. 2015)).

Data analysis

The design of this study was descriptive, and all data analyses were performed with the statistical software R (R Core Team 2021). Mass Array data were analysed as described previously (Davidoff et al. 2016). Sensitivity and specificity were calculated treating the MassArray results as the gold standard. Sensitivity was calculated as the proportion of those who had failed the PIC tests out of those who had a genotype that conferred red-green colour vision deficiency on the MassArray. Specificity was calculated as the proportion of those who passed the PIC tests out of those who had a genotype that conferred normal red-green colour vision on the MassArray. Fisher’s exact test was used to calculate p-values when comparing Hardy–Weinberg equilibrium expected versus identified frequencies. Significance level was set at 0.05.

Results

MassArray genotyping

Figures 1 and 2 show the estimated percentage of total X-chromosome opsins that are L genes plotted as a function of the percentage of total X-chromosome opsins that have the ‘first gene’ promoter allele of all the gene promoter fragments for males and females, respectively. T and L are the
theoretical average numbers of total opsin genes and of L opsin genes per X-chromosome, respectively. The visualization of the results makes it easy to separate out those who have opsin genes conferring normal from red-green deficient colour vision. Those with more than one gene (T genes conferring normal from red-green deficiency) are grouped towards the upper left corner of the plot (L = 2, T = 2, 3 or 4). Those conferring a protan colour vision deficiency will have no L opsin genes and are grouped towards the lower right corner (L = 0, T = 1).

Table 1 shows the classification of participants based on MassArray results presented in Figs. 1 and 2 with 78.6% of all participants found to have one L opsin gene followed by one or more M opsin genes. Those who fall above the unity line will have extra L opsin genes (L > 1). For females only, if the first two genes in the array are OPN1LW followed by OPN1MW, on both X-chromosomes, then they are expected to have normal colour vision. Similarly, if the first two genes on both X-chromosomes are OPN1LW, then they are expected to have a deutan type deficiency. Deutan carrier status is inferred if there is an extra L opsin gene on only one of a female’s X-chromosomes. However, because the genotyping does not provide information about the order of the genes on the X-chromosome, it cannot distinguish between females with ‘extra’ L genes who are normal versus deutan carriers. Proton carriers are expected to have fewer L opsin genes and will fall below the unity line. An individual who lacks L opsin genes (L = 0) will fall along the x-axis and be proton. Individuals who only have one gene (T = 1) are dichromats. Five males (2.6%) were found to have opsin genes resulting in colour vision deficiency, three deutan (L = 2, T = 2), one deutan with a promoter mutation and one protonope (L = 0, T = 1). The deutan-suspect group

| Colour vision status | Female % of females | Male % of males | Total % of all |
|----------------------|---------------------|----------------|----------------|
| Normal               | 203                 | 154            | 357            | 78.6 |
| Normal extra L       | 8                   | 4.1            | 8              | 1.8  |
| Protonope            | 1                   | 0.5            | 1              | 0.2  |
| Deuteranomalous      | 3                   | 1.6            | 3              | 0.7  |
| Promoter mutation (deutan) | 1 | 0.5 | 1 | 0.2 |
| Deutan suspect       | 28                  | 10.7           | 26             | 13.5 | 11.9 |
| Proton carrier       | 5                   | 1.9            | 5              | 1.1  |
| Deutan carrier       | 20                  | 7.7            | 20             | 4.4  |
| No MassArray data    | 5                   | 1.9            | 5              | 1.1  |

Percentages indicated in italic.
The shaded rows are colour deficient males.

(11.9% of the total) includes males with multiple L opsin genes on the X-chromosome and females with multiple L genes on one or both X-chromosomes, a total of 11.9% (Figs 1 and 2; L > 1, T > 2).

Comparing colour vision tests with MassArray genotyping in males

Table 2 shows specificity, sensitivity and predictive values if the MassArray results are considered as a proxy gold standard in males (see Table 5 for females). Eleven deutan-suspect males made no errors on PIC tests. Table 2 shows the calculations for individuals excluding all the deutan suspects who made no errors on PIC tests (left side) or including 50% of them (right side).

| CV Test results | MassArray genotype |
|-----------------|--------------------|
|                  | CVD Positive | CVD Negative | CVD Positive | CVD Negative |
| Ishihara ≥3 errors | Positive | 10 | 3 | 0.77 | 0.06 | 0.50 | 0.98 | 0.40 | 0.98 |
|                  | Negative    | 10 | 170 | 0.06 | 15 | 165 | 0.08 | 0.07 |
|                  | HRR ≥2 errors | Positive | 10 | 5 | 0.67 | 10 | 5 | 0.67 | 0.08 |
|                  | Negative    | 10 | 168 | 0.06 | 15 | 163 | 0.08 | 0.07 |
|                  | Ishihara & HRR combined | Positive | 10 | 0 | 1.0 | 10 | 0 | 1.0 | 0.08 |
|                  | Negative    | 10 | 173 | 0.05 | 15 | 168 | 0.08 | 1.0 |
|                  | Sensitivity | 0.50 | 1.0 | 0.40 | 1.0 |
|                  | Specificity | 0.08 | 0.07 | 0.08 | 0.08 |

The Sensitivity and Specificity values as indicated in the bottom row (bold and italics)

If only one of the PIC tests is used for screening, employing standard criteria, sensitivity and specificity were 0.50 and 0.98/0.97. The combined criteria for failing the PIC tests did not change sensitivity, but specificity improved to 1.0. The individuals in the group of deutan suspects have an opsin array with two L genes and one or two M genes. There is an indication from Davidoff et al. (2016) that half of deutan suspects who make no errors on the HRR may be assumed to have mild deuteranomaly. If half of the deutan suspects who make no errors on either test are included in the group with positive MassArray Genotype (right half of Table 2), sensitivity drops to 0.40 but specificity stays the same.

Table 2. Specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) with MassArray result as the proxy gold standard for identifying red-green colour vision deficiency (CVD) in males, either excluding all the deutan suspects (left side) who made no errors on pseudoisochromatic (PIC) plate tests or include 50% of them (right side).
Promoter mutation

Table 3. Classification of males (n = 193) when combining MassArray and pseudoisochromatic (PIC) plate test results.

| MassArray status | Errors on PIC tests combined | Errors on each PIC test | No errors |
|------------------|-----------------------------|-------------------------|-----------|
|                  | Red-green CVD | Undefined | Ishihara | HRR |                  |
| Normal           | ≥5 % | <5 % | ≥1 % | ≥1 % | 0 % |
| Normal extra L   | 41 % | 21.2 % | 37 % | 19.2 % | 8 * | 4.1 | 112 % | 58.0 % |
| Protanope        | 1 % | 0.5 % | 1 % | 1.0 % | 1 % | 0.5 | 1 % | 0.5 | 6 % | 3.1 |
| Deuteranomalous  | 1 % | 1.0 % | 1 % | 1.0 % | 1 % | 0.5 | 1 % | 0.5 | 6 % | 3.1 |
| Promoter mutation | 1 % | 0.5 % | 1 % | 0.5 | 1 % | 0.5 | 6 % | 3.1 | 11 % | 5.7 |
| Deutan suspect   | 6 % | 3.1 % | 15 % | 7.8 % | 6 % | 3.1 | 11 % | 5.7 | 129 % | 63.7 |

* One individual made one error on the diagnostic plates in addition to 2 errors on the screening plates of the HRR, but no typical errors on the Ishihara. All others made 1–2 errors on the HRR screening plates, of whom three also made 1–2 typical errors on the Ishihara.
* Half of the deutan suspects who made no errors are expected to be red-green colour deficient.

Table 4. Grading of red-green colour-deficient males who also made Rayleigh matches [match midpoint (MP)/matching range (MR)] with estimated spectral separation of the residual L/M photopigments (Δλ). Types of error on HRR are categorized as N = normal, D = deutan, P = protan.

| Rayleigh MP/MR | Errors on Ishihara | Errors on HRR/Type/Grade | Opsin gene array from MassArray | Δλ |
|----------------|---------------------|--------------------------|---------------------------------|-----|
| Deuteranomalous | 17.2/1.1 | 1 | 0/N | LL | Unknown |
| Deuteranomalous | 17.8/2.9 | 1 | 11/Medium | LLLM | Unknown |
| Deuteranomalous | 17.8/8.1 | 15 | 10/Mild | LLM | 2.5 |
| Deuteranomalous | 16.2/10 | 12 | 12/D/Medium | LLM | 2.5 |
| Deutanoppe | 36.5/73 | 14 | 13/D/Strong | LLLM | 0 |
| Protanope | 39.4/38.7 | 13 | 18/P/Strong | M | 0 |

- Typically associated with deuteranomaly (Table 4 and marked with footnote a in Table 3). Nine other males, who on MassArray were classified as deutan suspects, made 1–4 typical errors on the PIC tests, and it is very likely they have mild deuteranomaly. When combining the information obtained from MassArray and including these nine deutan suspects, 10.4% of males were found to be red-green colour deficient (9.8% deutan and 0.5% protan). If half of the deutan suspects who make no errors on PIC tests also are mild deutans and are included in the frequency estimate, then 13.2% of males were found to be red-green colour deficient (12.7% deutan and 0.5% protan). In either case, only 5.2% have been identified as red-green colour deficient with the PIC tests.

Table 4 shows the Rayleigh match midpoints (MP) and matching ranges (MR) set by the five males who were identified as red-green colour deficient on the PIC tests. The table also includes the one male, who made only one typical error on the Ishihara and none on the HRR, but he made a deuteranomalous match. Rayleigh MP and MR for those with normal opsin genes per array in the MassArray analysis.

The shaded rows are colour deficient males. Percentages indicated in italic.

Table 3 summarizes the classification when combining MassArray and colour vision test results for males. The first column shows MassArray classification, the second, third and last columns show the colour vision test results combining the results from both PIC tests. Those who made five errors or more combined on the Ishihara and the HRR tests were classified as red-green deficient, and 10 males were identified to be red-green colour deficient. Three had opsin gene arrays associated with deutan or protan deficiency, six were deutan suspects and one had promoter mutation conferring deuteranomaly. The two middle columns show the number of participants who made one error or more on each of the two PIC tests, as one male with MassArray conferring red-green colour vision deficiency made only one error on the Ishihara test but made a Rayleigh match midpoint and range typically associated with deuteranomaly (Table 4 and marked with footnote a in Table 3). Nine other males, who on MassArray were classified as deutan suspects, made 1–4 typical errors on the PIC tests, and it is very likely they have mild deuteranomaly. When combining the information obtained from MassArray and including these nine deutan suspects, 10.4% of males were found to be red-green colour deficient (9.8% deutan and 0.5% protan). If half of the deutan suspects who make no errors on PIC tests also are mild deutans and are included in the frequency estimate, then 13.2% of males were found to be red-green colour deficient (12.7% deutan and 0.5% protan). In either case, only 5.2% have been identified as red-green colour deficient with the PIC tests.

Comparing colour vision tests with MassArray genotyping in females

Table 5 shows specificity, sensitivity and predictive values when the MassArray results are considered as a proxy gold standard in females (see Table 2 for males). If only one of the PIC tests is used for screening, employing standard criteria, the Ishihara had poor sensitivity compared with the HRR. The combined criteria for failing the PIC tests gave sensitivity and specificity of 1.0 and 1.0.

Table 6 summarizes the classification when combining MassArray and colour vision test results for females (presented in the same way as for males in Table 3). Three of 261 females failed the PIC tests, of whom one had a normal opsin gene array. She reported to be a carrier and had the condition of vitamin D-resistant rickets, and we suspect this might be the reason she failed both PIC tests. More than 50% of deutan suspects and deutan or protan carriers identified with MassArray and nearly 25% of females with normal opsin genes made 1–4 typical errors on the diagnostic plates in addition to 2 errors on the screening plates of the HRR, but no typical errors on the Ishihara. All others made 1–2 errors on the HRR screening plates, of whom three also made 1–2 typical errors on the Ishihara.
Table 5. Specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) with MassArray result as the proxy gold standard for identifying red-green colour vision deficiency (CVD) in females.

| CV Test results | MassArray genotype | CVD Positive | CVD negative |
|-----------------|--------------------|--------------|--------------|
| Ishihara ≥3 errors | Positive | 1 | 2 | 0.33 PPV |
|                  | Negative | 1 | 257 | 0.00 NPV |
| HRR ≥2 errors | Positive | 2 | 13 | 0.13 PPV |
|                  | Negative | 0 | 246 | 0.0 NPV |
| Ishihara & HRR combined | Positive | 2 | 1 | 0.67 PPV |
|                  | Negative | 0 | 258 | 0.0 NPV |

The Sensitivity and Specificity values as indicated in the bottom row (bold and italics)

errors on PIC tests, more frequently on the Ishihara than the HRR.

Hardy–Weinberg equilibrium estimates

Table 7 shows the expected and identified frequency of females with colour vision deficiency (left-hand-side of table) and carriers (right-hand-side of table) based on the frequency of colour vision deficient males. The expected frequency of females with colour vision deficiencies was calculated with the equation \( p^2 + d^2 \) and of female carriers was calculated with the Hardy–Weinberg equilibrium (HWE) equation \( 2p(1-p^2) + 2d(1-d^2) \), where \( p \) and \( d \) are the frequency of protan and deutan deficiencies in males, respectively (Sharpe et al. 1999). The first row is based on the conservative calculation that 10.4% of males are red-green colour deficient. The second row is based on the calculation that 13.2% of males are red-green colour deficient, including half of the deutan suspects who made no errors on PIC tests.

The identified frequency of female carriers is based on results from MassArray and family history. From MassArray analysis, 1.9% were identified as protan carriers. In addition, 3.4% were identified as deutan carriers and 3.8% as deutan suspects/carriers and made errors on PIC tests (Table 6). From self-reported family histories, another 7.9% were identified as carriers with normal cone opsin arrays. The first row is based on this conservative calculation. The second row also includes half of the female deutan suspect and carriers who made no errors on the PIC tests and assumes that all protan carriers were detected by MassArray. The difference in expected and identified frequencies of females with colour vision deficiency and carriers is small and non-significant in both cases.

Discussion

This is the first report comparing MassArray results from cone opsin genetics with the Ishihara PIC test results for males and females. The Ishihara and HRR PIC tests, using the standard criteria for failing the tests, either alone or in combination, have very poor sensitivity; only 50% of the males with opsin gene arrays conferring red-green colour vision deficiency were identified. Combining MassArray with either PIC test improves the sensitivity considerably, identifying >97% of colour-deficient males and misidentifying 1% of colour normal males. These findings match that reported by Davidoff et al. (2016) when combining MassArray with a new minimalistic paper and pencil test alone. The HRR alone identifies all the red-green colour-deficient females, even one with an acquired deficiency. None of the tests are designed to identify carriers, but half of the carriers made several errors on the PIC tests, more frequently on the Ishihara than the HRR, confirming previous reports (for a review see Ref. (Dees & Baraas 2014)). The results in this study show that combining MassArray genotyping and PIC tests is more reliable in correctly identifying red-green colour-

Table 6. Classification of females (n = 261) when combining MassArray and pseudoisochromatic (PIC) plate test results.

| Colour vision status | Errors on PIC tests combined | Errors on each PIC test |
|----------------------|-------------------------------|-------------------------|
|                      | Red-green                     |                        |
|                      | CVD                           | Undefined              |
| MassArray            | ≥5 %                          | <5 %                   |
| Normal               | 1* 0.4                        | 66 25.3                |
| Deutan suspect/carrier | 2 0.8                        | 10 3.8                 |
| Proton carrier       | 2 0.8                        | 2 0.8                  |
| Deutan carrier       | 9 3.4                        | 5 1.9                  |
| No MassArray         | 3 1.1                        | 88 33.7                |
|                      | >0.1 %                        | 64 24.5                |

|                      | Ishihara ≥1 %                 | HRR ≥1 %               | No errors |
|                      | 64 24.5                       | 88 33.7                |

\( ^1 \) This female reported to be a carrier and has the condition Hypophosphatemic rachitis (not medicated within the prior 3–4 years).

\( ^1 \) Those with missing MassArray have been included in all calculations assuming they all have MassArray results consistent with normal colour vision. Percentages indicated in italic.

The shaded rows are colour deficient males.
deficient individuals and carriers than any single PIC test alone. In normal trichromacy, the X-chromosome array has typically one copy of the L opsin gene followed by two or more copies of the M opsin gene. An important feature of the MassArray is that it detects rearrangements of the opsin genes that delete either the normal L or M opsin gene. In some cases, the rearrangement produces a dichromat, in others an anomalous trichromat. All anomalous trichromats identified by the MassArray analysis are missing either a normal L photopigment or a normal M photopigment, with the MassArray providing an estimate of the spectral separation of the residual L/M photopigments. This separation has been shown to be correlated with the severity of anomalous colour vision (Neitz et al. 1996).

Because MassArray genotyping is a direct measure of the biological basis of colour vision, it makes the MassArray unparalleled in distinguishing dichromats from anomalous trichromats. The MassArray test, however, cannot distinguish some deuteranomalous individuals from ‘deutan suspects’. If someone passes both PIC tests, but has a MassArray result that does not distinguish between a deutan suspect and a deutan deficiency, sequencing the L and M cone opsin genes and their regulatory regions would allow to separate the two, but this is time-consuming and costly (Neitz & Neitz 2011). In some cases, an additional colour vision test, ideally an anomaloscope test, would be required to obtain an accurate diagnosis. When the anomaloscope is not available, MassArray combined with PIC tests identifies potential colour-deficient individuals that would go undetected by a PIC test alone (Table 3, footnote a). The judicious use of the information from the combined testing would never result in a person being misidentified as colour deficient just because they do not have a normal genetic makeup.

The Hardy–Weinberg equilibrium estimates of frequencies of colour vision deficiency and carriers in the female population closely matched our reported frequencies for females (Table 7), confirming the validity of the main findings in our study. This lends support to the reported frequencies of protan and deutan deficiencies in the male population, underlining the problem with conducting only one PIC test for the identification of colour-deficient individuals. Employing only one PIC test, either the HRR or the Ishihara, to define whether a person is normal or colour deficient should be avoided. But what is the significance of misidentifying mild red-green deficiencies as ‘normal’, as can happen when only one PIC test is used? An inadequate screening of a child may lead to education paths and career expectations, which are then unduly closed off upon the chosen profession’s more thorough colour vision testing. Alternatively, an inadequate screening of a colour-deficient adult may lead them to conduct work within an environment where medical fitness standards require normal colour discrimination for safe operation. The degree of risk depends on the context and whether further testing with other tests and/or a Rayleigh anomaloscope are available. Preferably both the HRR and the Ishihara PIC tests should be employed adopting a no-error criterion for the identification of colour-deficient individuals. However, to be certain if a person has normal colour vision, historic data show that Rayleigh anomaloscope is needed (Waaler 1927). Anomaloscope, however, is time-consuming, requires a trained examiner and is difficult to perform on children. Anomaloscopes are also rarely available in a clinical practice. Providing a saliva sample for MassArray analysis is, by comparison, easy and fast and can even be performed by children.

Rayleigh anomaloscopy is also poor in predicting more general aspects of colour perception such as colour discrimination (Barbur et al. 2008) and surface-colour judgements with natural scenes (Baraas et al. 2006; Baraas et al. 2010). Thus, whether abnormal opsin genes (that confer a red-green deficiency that cannot be reliably identified with two PIC tests) would manifest in a phenotypic difference of more general colour judgements when comparing with those with normal opsin genes, cannot be known if the genotype remains unknown. This emphasizes the importance of patient communication about the risk that mild red-green colour deficiencies can be misidentified as normal trichromacy if a clinical practice uses one PIC test only for the identification of colour vision deficiencies.

### Study limitations and strengths

Sequencing of regulatory regions would be needed to be able to ascertain if an individual who has a normal opsin gene array but fails PIC tests, has any other opsin gene mutation causing his red-green deficiency (Neitz & Neitz 2011). This was not carried out in this study because extracted DNA from saliva samples is known to yield too little DNA for this kind of analysis. Given the limitations of MassArray in detecting all abnormal opsin genes, it is still justified to treat the results as a proxy gold standard as 98.6% of individuals with genes known to cause vision deficiency would be identified with a genetic MassArray test alone (Davidoff...
et al. 2016). In doing so, it offers novel and insightful information about the relationship between the genotype and the phenotype, the latter as depicted by the Ishihara and the HRR tests.

A further limitation of the study is that Rayleigh anomalouscopic was not performed on all participants; however, the results confirm that the HRR does a reliable job of typing and grading red-green colour deficiencies when comparing with the results from the Rayleigh match (Table 4) (Bailey et al. 2004; Davidoff et al. 2016). Just relying on self-reported family histories and not having actual knowledge about the carrier status of females is another limitation; however, the Hardy-Weinberg estimates indicate that these histories approximate reality. A strength is that the participants comprised a representative sample of the Norwegian population for the given age group (Hagen et al. 2018).

Conclusion
Combining the Ishihara with MassArray appears to be sufficient for identifying red-green colour-deficient males, but not females. Including the HRR in the battery of tests allows for the identification of red-green colour-deficient females as well as grading of the deficiency. In general, even when the genotype is not known, as a minimum both the HRR and the Ishihara PIC tests should be completed, and the results combined, in the identification of red-green colour deficiencies. Further studies are required to understand the phenotypical implications of cone opsin genes that confer red-green colour vision deficiencies that cannot be reliably identified with standard PIC tests. Having this knowledge will allow for advances in understanding opsin gene variation and composition, as well as the consequences it may have on health and disease; ranging from myopia susceptibility (Greenwald et al. 2017; Hagen et al. 2019) to susceptibility for age-related changes, as well as the risks associated with colour vision deficiencies in the transport industries.

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