Word Count: 2005

Title: Estimating the live birth prevalence of recurrent 22q11.2 deletions from population-based newborn screening

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Abstract

Word Count: 211

Background: Pathogenic 22q11.2 deletions are an important cause of developmental delay and multimorbidity with significant burden of disease and early mortality. However, variable expression of the associated 22q11.2 deletion syndrome (22q11.2DS) contributes to under-recognition, with diagnostic delays common and prevalence uncertain. We sought to provide a contemporary estimate of live birth prevalence of typical 22q11.2 deletions using a population-based newborn screening (NBS) sample.

Methods: Using DNA available from 30,074 Ontario NBS dried blood spot samples and standard methods, we prospectively screened for 22q11.2 deletions with multiplex quantitative PCR (qPCR) assays, and independent confirmatory studies. Available clinical data, including qPCR of T-cell-receptor excision circles (TRECs, used in NBS for severe combined immunodeficiency), enabled comparisons between samples with and without 22q11.2 deletions.

Results: The minimum estimated prevalence was 1 in 2313 live births (95% CI 1/1352–1/4344) based on NBS samples with confirmed 22q11.2 deletions (n=13). Of term singletons, samples with 22q11.2 deletions had significantly younger maternal age (p=0.008), smaller birthweight for gestational age (p=0.0009), and lower TREC levels (p<0.0001).

Interpretation: The prevalence and clinical findings support the potential public health importance of early identification of 22q11.2DS. Multi-centre NBS studies, with linkage to paediatric data, are needed to identify factors that may affect live birth prevalence and infant mortality related to 22q11.2 deletions.

Keywords: DiGeorge syndrome, velocardiofacial syndrome, epidemiology, newborn screening, copy number variation, genomic disorder

Introduction

22q11.2 Deletion Syndrome (22q11.2DS, OMIM 188400/192430), previously called DiGeorge or velocardiofacial syndrome, is an important genetic condition associated with recurrent 22q11.2 microdeletions and highly penetrant expression.[1] Features include developmental delay, intellectual disability, congenital cardiac and/or palatal anomalies, paediatric immunodeficiency, and treatable endocrinological and neuropsychiatric conditions. Variable presentation, often without major anatomical anomalies, contributes to clinical under-recognition and diagnostic delay.[1,2]

There are no contemporary population-based live birth prevalence estimates for 22q11.2 deletions based on newborn screening (NBS) data. Reported prevalence estimates vary widely, most commonly stated as 1 per 3000 to 1 per 6000 live births.[1] Dating back to 1996,[3] previous estimates have used multiple strategies, including ascertainment from birth defects registries,[3,4] infants with congenital cardiac disease,[4,5] or clinically-indicated genetic testing results.[6,7] One study used 25,704 NBS samples selected from individuals born 1981 to 2005 to retrospectively identify 22q11.2 deletions, but excluded neonatal and early infant deaths.[8]

NBS programs using T-cell receptor excision circles (TRECs) for identification of severe combined immunodeficiency (SCID) can detect some individuals with 22q11.2DS where there is neonatal immunodeficiency,[9,10] but phenotypically-based methods are unlikely to be sufficient for population-wide detection of pathogenic deletions with such variable expression.[10,11] Given the morbidity and mortality associated with 22q11.2DS that extends throughout the lifespan,[1,12-14] it has been proposed that 22q11.2 deletions be added to NBS panels, a plan endorsed by families of affected individuals.[15] In addition to NBS considerations, estimates from prenatal

studies and technological advances in prenatal screening for 22q11.2 deletions have increased the urgency of determining the current live birth prevalence.[16,17]

We sought to obtain a minimum estimate of the live birth prevalence of typical 22q11.2 deletions using contemporary population-based NBS data. We also examined available clinical data including TREC results.

Methods

Patient samples

There were 30,074 anonymized dried blood spot (DBS) samples available for study collected by Newborn Screening Ontario between January 2017 and September 2018 (99.8% in 11 of these 21 months), and corresponding to 11.7% of all newborns born in Ontario, Canada during this time period. This study was approved by the Children's Hospital of Eastern Ontario Research Ethics Board (REB) and the Centre for Addiction and Mental Health REB. Per REB approval guidelines for DBS samples, minimal clinical data for these newborns were available.

Laboratory investigations and clinical variables

Residual DNA from the TREC quantitative real-time polymerase chain reaction (qPCR) assay, a part of Ontario's NBS program for SCID,[18] was available for these 30,074 samples and was used to screen for the most common pathogenic 22q11.2 deletions (Figure 1).[1] The primary 22q11.2 deletion qPCR screening assay comprised primers and probes for three 22q11.2 deletion region genes (Figure 1): *UFD1L* and *COMT* (low copy repeat LCR22A-LCR22B region), and *CRKL* (LCR22C-LCR22D region), with *RPPH1* used as a reference gene for appropriate DNA extraction and relative quantification (Supplemental Methods, Tables S1, S2). For each probe, the relative quantification (RQ) was calculated and a cut-off defined using the area under the Receiver Operating Curve (Supplemental Methods, Table S3). A screen-positive sample was

defined as one with a putative deletion of all three 22q11.2 region probes, or of both *UFD1L* and *COMT* probes. Screen-positive samples with sufficient DNA for an additional DBS punch were then subjected to a secondary qPCR screening assay using the same reference probe but different 22q11.2 probe (*TBX1*, Supplemental Methods, Table S3).

The primary screen-positive samples that also had a *TBX1* RQ value below (or near) an established cut-off were prioritized for standard multiplex ligation-dependent probe amplification (MLPA; MRC Holland, Amsterdam, Netherlands) assays; six screen-negative samples served as controls for MLPA normalization (Supplemental Methods). Subsequently, samples having *UFD1L* and *COMT* RQ values closest to those of samples with MPLA-confirmed 22q11.2 deletions were prioritized for MLPA assays. Samples were deemed to have a confirmed 22q11.2 deletion if they screened positive on the initial three-probe qPCR assay and MLPA determined presence of a common or proximal nested 22q11.2 deletion (Figure 1).

Methodological details including DNA extraction and creation of DBS quality control material for all qPCR and MLPA assays are provided in Supplemental Methods. With respect to clinical variables available, these were confined to maternal age, newborn sex, birth weight, gestational age, neonatal transfusion status, and neonatal feeding type, each with varying number of usable data points. All 30,074 samples had a TREC value from the NBS SCID assay.

Statistical analyses

We calculated a minimum live birth prevalence estimate of the 22q11.2 deletion by dividing the number of NBS samples with MLPA-confirmed 22q11.2 deletion by the total number of NBS samples screened. 95% confidence intervals (CI) for prevalence were calculated based on the Poisson distribution for rare events. For the subgroup of singleton newborns born at term, defined as ≥37 and <42 weeks gestational age,[19] we compared TRECs and other available

clinical variables between those with a confirmed 22q11.2 deletion and the remaining population-based sample (Supplemental Methods) using $\chi 2$ or Fisher's exact test for categorical variables, and Mann-Whitney-*U* with Monte Carlo estimate tests for non-parametric continuous variables. 95% CI for proportions were calculated using the binomial distribution. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC). We defined statistical significance as $p<0.05$, two-tailed.

Results

Estimated live birth prevalence of the 22q11.2 deletion

Screening and confirmatory assay results provided a minimum live birth 22q11.2 deletion prevalence estimate of 1/2313 (95% CI 1/1352–1/4344) based on 30,074 Ontario NBS samples. The 13 samples with screen-positive results on the primary qPCR assay and MLPA confirmation comprised ten (76.9%) with the common LCR22A-LCR22D 22q11.2 deletion, and three (23.1%) with proximal nested deletions (two LCR22A-LCR22B, one LCR22A-LCR22C) (Figure 1).

Clinical variables

All thirteen NBS samples with a 22q11.2 deletion were singleton births; one was preterm. Within the total NBS samples with singleton term births (n=26,448), most had clinical data available. Those with a 22q11.2 deletion had a significantly younger median maternal age (25, Q1-Q3 24-30 years vs. 32, Q1-Q3 28-35 years, Z=-2.59, p=0.008; Figure 2A, Table S4). Those with a 22q11.2 deletion were also enriched for low (<10th percentile) birth weight for gestational age (n=6, 50.0%, 95% CI 21.1-78.9% vs. n=2869, 10.9%, 95% CI 10.5-11.3%, p=0.0009; Figure 2B, Table S4). A complex neonatal feeding type was also more likely in the 22q11.2 deletion subgroup (n=2, 18.2%, 95% CI 2.3-51.8% vs. n=183, 0.8%, 95% CI 0.7-0.9%, p=0.003; Table S4); there were no significant differences for other variables examined (Table S4).

TREC values

Term singleton samples with a 22q11.2 deletion had significantly lower median TREC values (99.3 copies/3μL) compared with the remaining population-based samples (602.6 copies/3μL, $p<0.0001$). The 22q11.2 deletion subgroup was enriched in samples with <200 TREC copies/3μL ($p<0.0001$, Figure 3A); six (50.0%) met the initial clinical NBS SCID cut-off value of ≤100 TREC copies/3μL (Figure 3B), compared with 81 (0.3%) of the population-based samples ($p<0.0001$). In Ontario NBS, all samples meeting this initial cut-off proceed to a second, confirmatory TREC assay, run in duplicate, with a cut-off of ≤75 copies/3μL. Of the total 87 samples proceeding to this secondary assay, 11 met this clinical cut-off: one (8.3%) from the 22q11.2 deletion group, and ten (0.04%) from the remaining population-based group ($p=0.005$).

Interpretation

The minimum estimated prevalence of the 22q11.2 deletion in the Ontario NBS sample studied was 1 in 2313 live births. This is higher than previous prevalence estimates using different sampling methods, but remains in line with 22q11.2DS as a rare disease (defined as <1/2000).[1] 22q11.2DS has historically presented a significant diagnostic challenge for clinicians, with clinical diagnosis based on obvious congenital anomalies that do not predict the intellectual or neuropsychiatric outcomes of most concern to parents.[1,2] The vast majority of affected newborns would be expected to be born to unaffected parents.[1] However, improved paediatric care over many decades, and limited effects of the 22q11.2 deletion on reproductive fitness when major neuropsychiatric phenotypes are absent, could lead to increasing numbers of affected parents, often undiagnosed.[20]

In contrast to other studies purporting to provide population-based prevalence estimates for 22q11.2DS,[3-8,21] the strength of this study is the unselected contemporary NBS sample that

was uniformly screened using a standard NBS method (multiplex qPCR). Several previous studies used clinically ascertained samples with the 22q11.2 deletion,[3-7] thus may have underestimated the prevalence of 22q11.2DS, given its variable clinical phenotype that often does not include typical congenital anomalies.[1] Only two previous population-based studies based prevalence solely on molecular genetic data.[8,21] One was a Danish study that was restricted to residents at one year of age or older and retrospectively examined DBS banked over a 24.5 year period to 2005,[8] thus could not account for infant mortality.[12] The other was a Norwegian study restricted to newborns that required both parents to consent to participate in a genetic research study.[21] The 22q11.2 deletions identified in these two studies represent bookends of highest (n=7 in 25,704)[8] and lowest (n=1 in 12,252),[21] respectively, estimated live birth prevalence prior to the current study.

In the current study, as expected, the majority of 22q11.2 deletions spanned the full LCR22A-LCR22D region (Figure 1).[1] However, rarer proximal nested 22q11.2 deletions comprised three (23.1%) of the 13 confirmed 22q11.2 deletions, a higher prevalence than reported in large clinically ascertained samples.[1,22] This raises the possibility that nested 22q11.2 deletions may have a somewhat lower penetrance for typical anatomical features leading to clinical detection[2] compared to the common LCR22A-LCR22D deletion.[22]

In contrast to more familiar chromosomal abnormalities such as trisomy 21, the limited clinical data available for the samples in the current study show that pathogenic 22q11.2 deletions may be associated with earlier, not late, maternal age.[23] Also, we found that the 22q11.2 deletion may be associated with mild abnormalities of fetal growth, consistent with results from a retrospective study of adults with 22q11.2DS.[24]

Notably, the TREC results, while consistent with previous TREC-based NBS studies indicating enrichment of the 22q11.2 deletion amongst low values,[9,10] indicated that only a minority of all confirmed 22q11.2 deletion samples would be detected using a SCID-based NBS strategy. This finding was foreshadowed by results of a previous retrospective study,[11] and provides further support for developing genetically-based NBS for 22q11.2 deletions.

Future directions

The findings set the stage for future prospective studies to further refine prevalence estimates of high-impact 22q11.2 deletions, including the rarer proximal nested deletions. Large multi-centre NBS studies involving diverse jurisdictions could allow determination of factors that may affect 22q11.2DS prevalence, such as ethnicity and cultural factors, availability of prenatal screening[25] and reproductive technologies, etc. If ethics approval could be obtained, assessment of phenotypic data, details about newborns receiving an early clinical diagnosis of 22q11.2DS, determination of the inherited and *de novo* 22q11.2 deletion status, and ability to provide parents of newborns with the 22q11.2DS diagnosis after clinical lab confirmation, would offer substantially improved understanding about this important condition. A recent study of prospective mothers and previous reports would support such a study design.[15,25]

Limitations

The main limitation of this and other 22q11.2DS prevalence studies is the sample size. Larger, comparably ascertained samples are needed to refine live birth prevalence estimates and knowledge about associated clinical features. Nonetheless, the results complement previous estimates using other designs, e.g., those based on congenital physical features and clinical recognition,[3-7] and add to studies showing high prenatal prevalence of the 22q11.2 deletion (1/992)[16] and strong association with fetal loss (stillbirths and miscarriages).[26,27] In addition, due

to REB restrictions, there were minimal clinical data available, precluding our ability to learn about factors that could affect prevalence of the 22q11.2 deletion, or outcomes of these newborns, including whether and when any received a clinical diagnosis of 22q11.2DS. Further, this study was not designed as a methods study, thus did not include confirmatory assays for all 30,074 samples, nor the ability to calculate true positive and true negative rates, or evaluate the specific qPCR-based assays used, although we note that qPCR is already a standard method used in existing NBS programs.

Conclusion

This study provides the first ever contemporary live birth prevalence estimate for pathogenic 22q11.2 deletions. The results indicate that 22q11.2DS is one of the most common of rare genetic conditions. The clinical findings from this study and others support the public health importance of early – prenatal and neonatal – diagnosis.[1,12]

Data availability

Data sharing is precluded as per REB.

Acknowledgements

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Figure Legends

Figure 1.

Illustration of the commonly deleted ~3 Mb 22q11.2 deletion and the rarer proximal nested ~2 Mb and ~1.5 Mb 22q11.2 deletions, and the approximate positions of genes for probes used to detect these deletions: three primary screening qPCR probes (bold font, single asterisk), 15 genes for confirmatory MLPA studies (16 probes, including 2 at *TBX1* and flanking probes at *USP18* & *HIC2*), and one secondary screening qPCR probe (two asterisks). Also shown are the relative positions of the low copy repeat sequences (segmental duplications) that predispose this complex genomic region to *de novo* 22q11.2 deletion events at gametogenesis, and probes (N25 and TUPLE1) commonly used for targeted fluorescence *in situ* hybridization (FISH) studies that cannot determine the length of deletions. Note that clinical genome-wide microarray, the current standard for pathogenic copy number variation detection, provides such information. Cen = centromere. LCR = low copy repeat. Mb = megabase.

Figure 2.

A. Median maternal age and interquartile range for term singleton NBS samples, by 22q11.2 deletion status. Only term singleton samples with available data on maternal age together with newborn sex, gestational age, and birth weight, were included (Supplemental Methods). Vertical lines at the tops of the bar graphs indicate the first and third quartiles for maternal age. The 22q11.2 deletion group (n=11) had a significantly younger median maternal age compared with the remaining population-based group (n=26,000; Z=-2.59, p=0.008). See Table S4 for detailed results.

B. Percentage of term singleton samples with low (<10th %ile) birth weight for gestational age, by 22q11.2 deletion status. Only samples from singleton newborns born at term with available sex, gestational age, and birth weight data were included (Supplemental Methods). The 22q11.2 deletion group had a significantly higher proportion (n=6/12, 50.0%) with low birth weight for gestational age compared with the remaining population-based group (n=2869/26,306, 10.9%, p=0.0009). See Table S4 for detailed results.

Figure 3.

A. SCID screening results showing distribution of TREC copies/3 µL for term singleton NBS samples by 22q11.2 deletion status. The majority (7/12, 58.3%) of the 22q11.2 deletion group had TRECs <200 copies/3µL (~3rd percentile), while the greatest proportion of the remaining population-based group (50.3% of 26,436) had TRECs ≥ 600 copies/3µL.

B. Subset of term singleton NBS samples with lowest TREC values (<200 copies/3µL). Shown here are detailed distribution results for NBS samples with TRECs <200 copies/3µL, n=7 with a confirmed 22q11.2 deletion and 852 from the remaining population-based group. The dashed horizontal lines indicate where the scale changes for fine gradations: below 10 indicating single individuals, and above 10 indicating 10 individuals. Six (50.0%) of the overall 12 samples with a confirmed 22q11.2 deletion had TRECs ≤ 100 copies/3µL (left of the vertical dashed line), compared with 81 (0.3%) of the remaining population-based group (n=26,436). 100 TREC copies/3µL is the Ontario NBS cut-off for inclusion into a secondary, more accurate TREC assay for final SCID NBS reporting.

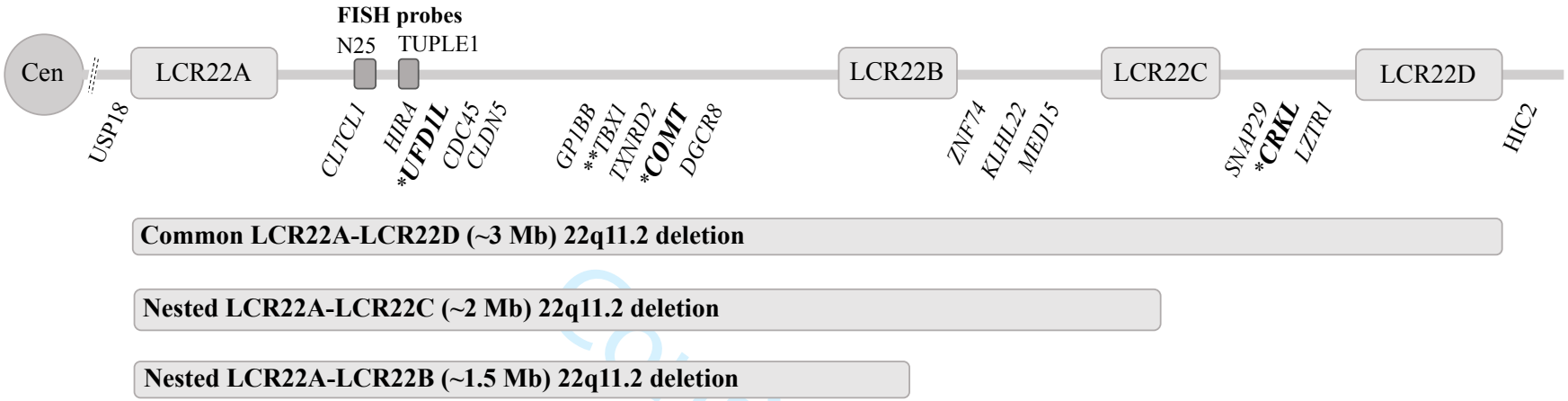

Figure 1.

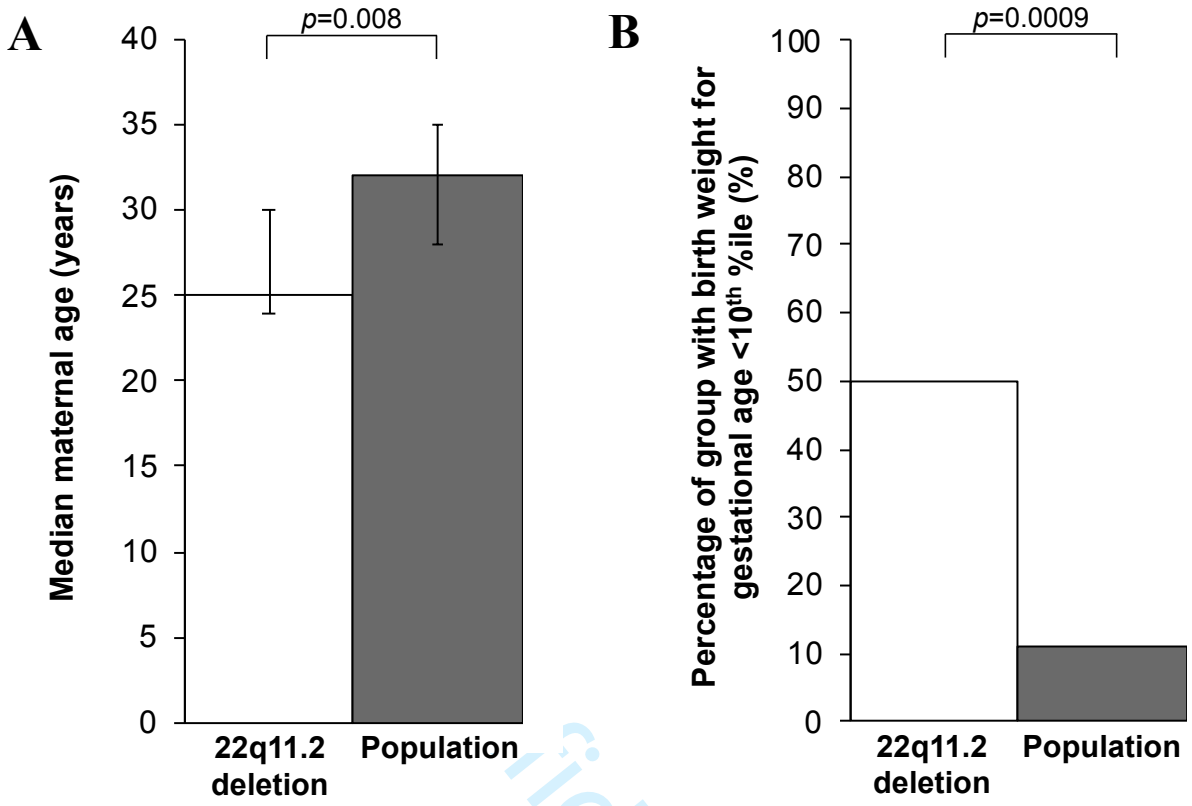

Figure 2.

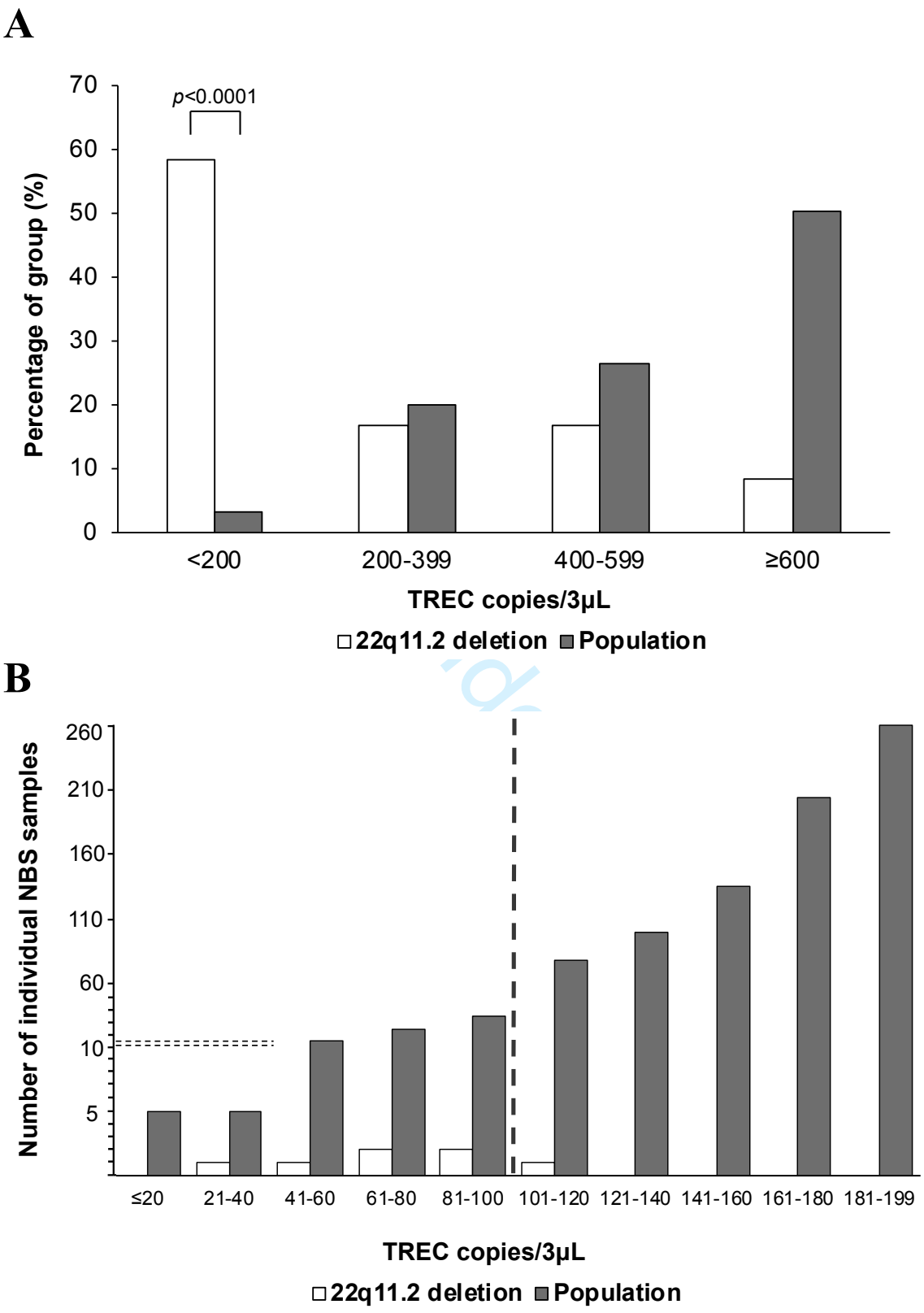
A
p<0.0001
Percentage of group (%)
70
60
50
40
30
20
10
0
<200
200-399
400-599
≥600
TREC copies/3μL
22q11.2 deletion
Population
B
Number of individual NBS samples
260
210
160
110
60
10
5
≤20
21-40
41-60
61-80
81-100
101-120
121-140
141-160
161-180
181-199
TREC copies/3μL
22q11.2 deletion
Population

Figure 3.

Supplemental Methods

Dried Blood Spot Quality Control Material

Dried blood spot (DBS) quality control material was created for use as positive and negative controls for all qPCR and MLPA assays. Immortalized B-cell lines from confirmed patients with the 22q11.2 deletion, GM07939, GM17938 and GM17942 (Coriell, New Jersey, USA), were washed twice in 1X PBS (Invitrogen, California, USA), before being mixed with saline-washed red blood cells (RBC) devoid of lymphocytes and mixed to a 50% hematocrit level with fetal bovine serum (Sigma, Missouri, USA). The mixture was then allowed to mix for > 1 hour on a Nutator (VWR, Pennsylvania, USA). Both the B-cell line-RBC mixture and whole blood from a wild-type (no 22q11.2 deletion) donor were applied to Whatman 903 Protein Saver cards (VWR) using a pipette (75 µl) and allowed to dry overnight at room temperature.

DNA Extraction from DBS for qPCR

There were 30,074 NBS DBS available for this study; the number of samples was determined by power analyses in the original study protocol and was also limited by funding. A single 3.2 mm disc was punched from each de-identified NBS DBS using the BSD 600 Plus (Luminex, Texas, USA) into a 96-well-U-bottom polypropylene microtiter plate (Corning, New York, USA). DNA was extracted on an NXp Span-8 liquid handler (Beckman Coulter, California, USA) using the following method. Each DBS was washed twice with 110 µl of 20 mM Tris-HCl pH 9.0, 0.5% Triton-X, and once with 110 µl of 20 mM Tris-HCl pH 9.0, each wash consisting of a ten-minute incubation at 37°C and 700 rpm on a heated microplate shaker. Then, 50 µl of Tris-HCl pH 9.0, 50 ng/µl yeast tRNA was added and plates were sealed with pierceable aluminum heat seal and sealer (Axygen, New York, USA) to prevent contamination and evaporation. DNA was eluted by incubation at 98°C for 40 minutes with shaking in a

VorTemp 56 (Labnet, New Jersey, USA). DNA plates were then stored at -20°C until completely frozen. DBS quality control material consisting of 22q11.2 deletion-positive and wild-type (i.e., 22q11.2 deletion negative) in-house made samples (as above) were punched and extracted along with population-based NBS samples on each 96-well plate for use in quantifying gene copy number, thus appearing four times each on a 384-well initial qPCR screening assay, and in duplicate on the secondary qPCR assay.

Quantitative PCR Assays

Relative quantification using a quadplexed 5'-hydrolysis qPCR initial assay was carried out in a 20 µl reaction volume on a 384-well plate (Life Technologies, California, USA). Each reaction contained 1X DurAmp Mastermix (Life Technologies), 400 nM for each gene specific primer (IDT, Iowa, USA), 200 nM for each gene specific probe (LGC BioSearch Technologies, Teddington, UK), i.e., for three 22q11.2 deletion region genes (Figure 1), *UFD1L, COMT, CRKL*, and a standard reference gene *RPPH1* (encoding RNase P), together with 8 µl of extracted DNA. The primers and probes for the primary screening assay are listed in Tables S1 and S2. On the initial qPCR assay, a screen-positive sample for the 22q11.2 deletion was defined as qPCR results consistent with the apparent deletion of all three 22q11.2 deletion region probes, or the apparent deletion of both *UFD1L* and *COMT* probes. This represented a refining of methodology for these three probes originally tested using spiked samples from individuals known to have 22q11.2 deletions (Wisconsin, unpublished data).

Screen-positive samples that had sufficient DNA for a second DBS punch were subjected to a secondary multiplexed qPCR assay run in a 20 µl total volume in a 96-well FAST qPCR plate (Life Technologies) that consisted of the following two target genes: *TBX1* (22q11.2 deletion region, Figure 1), and *RPPH1* (reference). Each reaction contained 1X DurAmp

Mastermix (Life Technologies), 900 nM for the *TBX1* primers, 250 nM for the *TBX1* probe[1] (Life Technologies, Cat#Hs01313390_cn FAM), 400 nM for the *RPPH1* primers, and 200 nM for the *RPPH1* probe. The primer and probe sequences for *RPPH1* are listed in Tables S1 and S2. The primer and probe sequences for *TBX1* are not listed as they are proprietary.

Primer and probe sequences were assessed against the human genome using the BLAST program to ensure 100% homology to only the sequence from which they were derived. The DurAmp master mix contains the ROX dye as an internal passive reference to which the reporter dye signal can be normalized by the software.

qPCR for both assays was performed using the Comparative Ct (ΔΔCt) mode on the Applied Biosystems ViiA 7 Real-Time PCR System using the following PCR cycling parameters: 1 cycle at 95°C (45 seconds) followed by 45 cycles at 95°C (30 seconds) and 60°C (1 minute 30 seconds).

qPCR Data Analysis

Relative quantification (RQ) of each gene was calculated using the ViiA 7 software v.1.2 (Life Technologies) by way of presenting the ratio of the 22q11.2 genes of interest, relative to the *RPPH1* reference gene from the de-identified NBS samples, compared to that of our wild-type quality control samples, following the ΔΔCT method.[2] The area under the Receiver Operating Characteristic (ROC) curve was calculated using non-parametric methods[3] to determine assay cut-off. The RQ cut-off values for a heterozygous 22q11.2 deletion are presented in Table S3.

Salting-Out Extraction of DNA from DBS for MLPA

Additional DBS punches were required for the confirmatory MLPA assay that used standard probes from the 22q11.2 region. A single 3.2 mm disc was punched (Wallac DBS

Puncher) and transferred to a 1.5 mL microtube followed by overnight lysis with 150 µl of STE buffer (1M Tris-HCl pH 8.5, 3M NaCl, 0.5M EDTA) plus 50 µl of proteinase K (20mg/mL; Sigma) at 56°C. The next day, 200 µl of lysis buffer (Nuclisens) was added and incubated at 56°C for 2 hours. Then, 250 µl of 7.5M NH_4OAc was added and the tube placed at -20°C for 2.5 hours, followed by centrifugation at 14.5k rpm for 15 minutes at room temperature. The supernatant (620 µl) was transferred to a fresh microtube and the following was added: 1 µl of glycogen (20mg/ml, Roche, Basel, Switzerland), 68.9 µl of 3M NaOAc, and 482.2 µl of isopropanol. The tube was mixed well and centrifuged at 14.5k rpm for 20 minutes at room temperature. The DNA pellet was washed with 1 mL of room temperature 70% ethanol and centrifuged at 14.5k rpm for 20 minutes at room temperature. The DNA pellet was left to air dry for up to 20 minutes and then resuspended in 10 µl of 10mM Tris pH 8.0 and heated at 55°C for 1-2 hours with gentle shaking every 20 minutes.

MLPA confirmatory assay

Screen-positive 22q11.2 deletion samples, in addition to six screen-negative samples used as controls for normalization, were assayed by MLPA with the SALSA® MLPA® Probemix P250-B2 "DiGeorge" MLPA kit according to the general protocol provided by the manufacturer (MRC Holland, Amsterdam, Netherlands). We used a maximum of 100 ng of DNA extracted from DBS. MLPA was performed on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher, Massachusetts, USA) and analysis was carried out with Coffalyser.Net software (MRC Holland). The dosage quotient (DQ) for heterozygous deletions was defined as $0.40 < DQ < 0.65$, as per MRC Holland guidelines for the kit.

A total of 63 samples had screen-positive initial qPCR assay results. Of the 50 other samples (i.e., excluding the 13 with confirmed 22q11.2 deletions), 32 (64%) had MLPA results

that confirmed absence of a 22q11.2 deletion (Figure S1) and one showed a smaller nested 22q11.2 deletion on MLPA (Figure S2D). Just one screen-positive sample had neither MLPA nor *TBX1* qPCR results available to indicate absence of 22q11.2 deletion (Table S3). MLPA results for the proximal nested LCR22A-LCR22B and LCR22A-LCR22C 22q11.2 deletions, the common LCR22A-LCR22D 22q11.2 deletion, and a small nested 22q11.2 deletion that did not meet study criteria are shown in Figure S2.

Clinical Variables

For the subset of NBS samples from singletons born at term with data available (n=26,318) we investigated the following clinical variables: sex, birth weight for gestational age, the proportion of newborns transfused at birth, the proportion of newborns with a complex neonatal feeding type, gestational age, and maternal age at birth. A minority of samples had missing data for any one of these variables, thus we have listed the sample sizes for each in the footnotes of Table S4. Main comparisons were between samples with a confirmed 22q11.2 deletion and the remaining population-based samples. We calculated the proportions of each group considered <3rd and <10th percentile for sex- and gestational age-corrected birth weights, based on a Canadian reference sample.[4] For the data on feeding available, those designated as "total parenteral nutrition (TPN)" or "nil per os" as the sole or one of several feeding types (e.g., breast and TPN), were classified as having a complex neonatal feeding type.

Supplemental Tables

Table S1. Primers used in the primary qPCR screening assay for 22q11.2 deletions

Primers	**Sequence 5' → 3'**
UFD1L FP	GTTTGACTTGGAAGTGGAGCAGCAG
UFD1L RP	GGAGCTCCCCTCAAGCTGAAAG
COMT FP	CAGTTGTGGTTACTTTCTGGAGAGAG
COMT RP	GGCCGCCCAGGAAGAC
CRKL FP	GAGAGAAGCCTGAAGAACAGTGG
CRKL RP	CTTTTCGACATAAGGGACAGGAAT
RPPH1 FP	TTGCCGGAGCTTGGAACAG
RPPH1 RP	ACCTCACCTCAGCCATTGAAC

FP: forward primer. RP: reverse primer.

UFD1L, COMT, and *CRKL* are from the 22q11.2 deletion region; *RPPH1* is a standard reference used for qPCR.

Table S2. Probes used in the primary qPCR screening assay for 22q11.2 deletions

Probe	**Sequence 5' → 3'**
UFD1L	AAGACAAAGAGCTGTCCCTGAGGG-FAM
COMT	CCTCCAGCTCCTGCAT-Quasar 670
CRKL	TGCCCGGAACAAGGATGGCC-Quasar 705
RPPH1	CTCACGGCCAGCGAAGT-VIC

Table S3: qPCR RQ cut-off values for 22q11.2 deletion region gene targets indicating a possible 22q11.2 deletion (i.e., screen-positive)

Probe	Target cut-off (i.e., RQ less than:)
Primary qPCR screening assay	
UFD1L	0.713
COMT	0.692
CRKL	0.776
Secondary qPCR screening assay	
TBX1	0.620

RQ = relative quantification.

See Supplemental Methods above for details of assays.

Note: The *TBX1* qPCR screening assay showed mixed results. Of 11 MLPA-confirmed 22q11.2 deletion samples with *TBX1* results, nine (81.8%) indicated a 22q11.2 deletion (RQ range 0.530-0.607), and two had results above the cut-off (RQ 0.657 and 0.650). For the remaining 50 primary screen-positive samples, *TBX1* results were available for 45, 44 (97.8%) of which indicated no 22q11.2 deletion (RQ range 0.621-1.314) and one confirmed to have no 22q11.2 deletion on MLPA despite a *TBX1* result (RQ 0.614) below the cut-off.

For just one of the total 63 primary screen-positive samples (Figure S1, where *UFD1L* and *COMT* results were below cut-offs, respectively, RQ 0.659, 0.664) there were neither MLPA nor *TBX1* results available.

Table S4: Demographic and clinical characteristics in term singleton newborns with a 22q11.2 deletion and in the population[a]

Demographic and clinical variables	22q11.2 deletion (maximum n=12)[b]		Population (maximum n=26,306)[b,c]			
	n	**% (95% CI)**	**n**	**% (95% CI)**		***p***
Male sex	4	33.3 (9.9-65.1)	13,467	51.2 (50.6-51.8)		0.22
Birth weight for gestational age (<10th %ile)[d]	6	50.0 (21.1-78.9)	2869	10.9 (10.5-11.3)		**0.0009**
Birth weight for gestational age (<3rd %ile)[d,e]	2	16.7 (2.1-48.4)	819	3.1 (2.9-3.3)		0.05
Neonatal transfusion[f]	0	0 (0-28.5)	12	0.06 (0.03-0.1)		1.00
Complex neonatal feeding[g,h]	2	18.2 (2.3-51.8)	183	0.8 (0.7-0.9)		**0.003**
	Median	**IQR**	**Median**	**IQR**	***Z***	***p***
Gestational age (weeks)	39.0	2.0	39.3	1.7	-1.40	0.16
Maternal age (years)[i,j]	25	6	32	7	-2.59	**0.008**

CI: Confidence intervals calculated using binomial proportions. IQR: Interquartile range

[a] Term was defined as 37≤gestational age<42 weeks.

[b] One individual of the 12 in the 22q11.2 deletion group and 109 individuals in the population group had DNA taken prior to 24 hours of age which may be considered a less than satisfactory sample as certain newborn screening tests may be less sensitive.

[c] Singletons born at term with available sex, birth weight, and gestational age data.

[d] Percentiles calculated based on a Canadian reference set of all singletons born in Canada between 1994-1996 (with the exception of Ontario).[3]

[e] Restricting to females, the proportion with a birth weight for gestational age <3rd %ile was significantly higher in the 22q11.2 deletion group (n=2 vs n=401, p=0.02).

[f] Neonatal transfusion data were available for n=11 from the 22q11.2 deletion group and n=21,699 from the population group.

[g] Complex neonatal feeding includes neonates who required total parenteral nutrition (TPN) or who were deemed "nothing by mouth" ("nil per os", NPO) as either their sole method of feeding or in combination with another feeding type.

[h] Neonatal feeding data were available for n=11 22q11.2 deletion individuals and n=24,414 population individuals. The n=2 individuals with 22q11.2 deletion were both deemed NPO at birth.

[i] Maternal age data was available for n=11 from the 22q11.2 deletion group and n=26,000 from the population group.

[j] One individual from the population group with a maternal age of >60 years was excluded due to a presumed data entry error.

Supplemental Figures

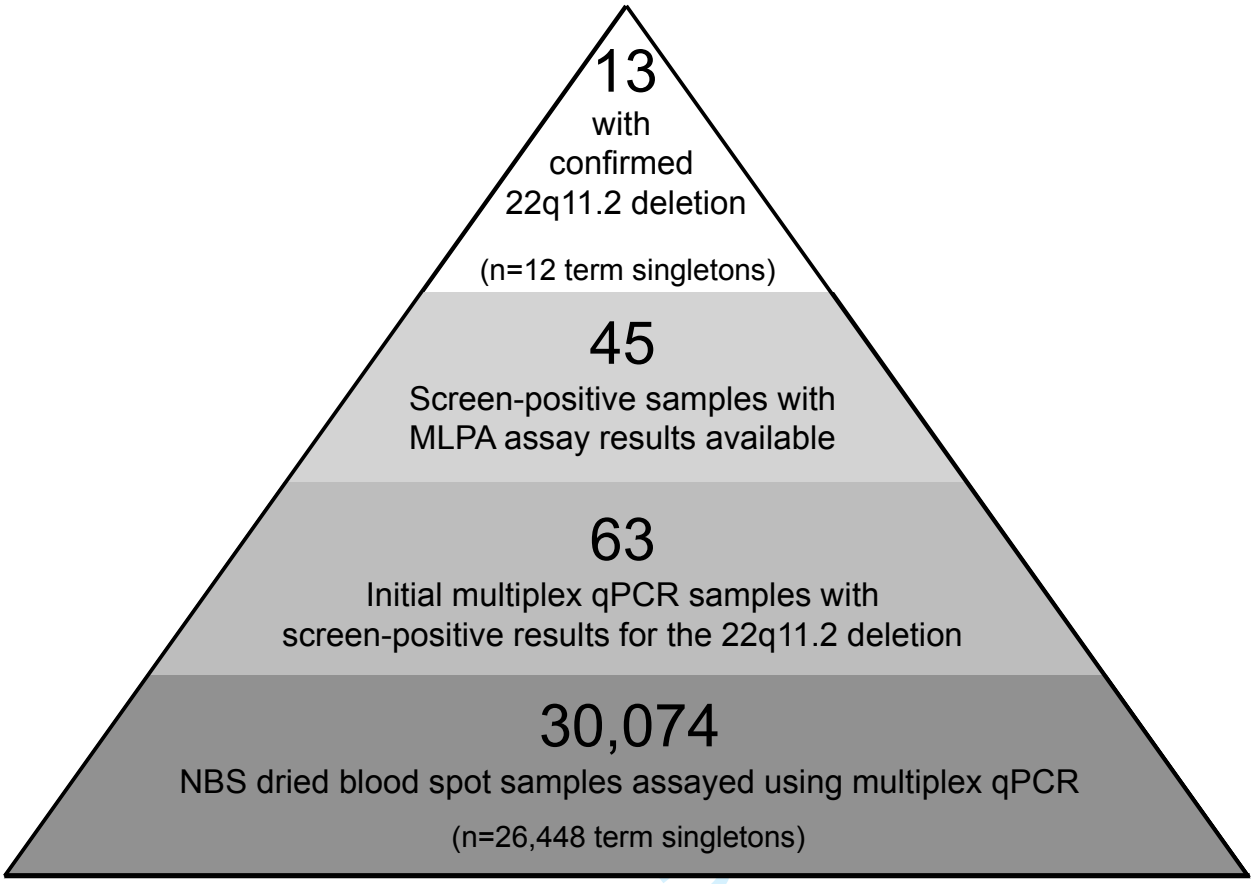

Figure S1. Sample and summary of screening and confirmatory results leading to a minimum live birth prevalence estimate for 22q11.2 deletions. Of the NBS samples that were screen-positive by the primary qPCR assay (n=63), 45 had an MLPA result available. Of these, 13 were confirmed to have a 22q11.2 deletion (10 with a common LCR22A-LCR22D deletion, 3 with a proximal nested deletion, see Figure 1); 32 did not meet pre-established criteria for a 22q11.2 deletion, 31 with MLPA-confirmed absence of the deletion and one with a small nested proximal 22q11.2 deletion (Figure S2D).

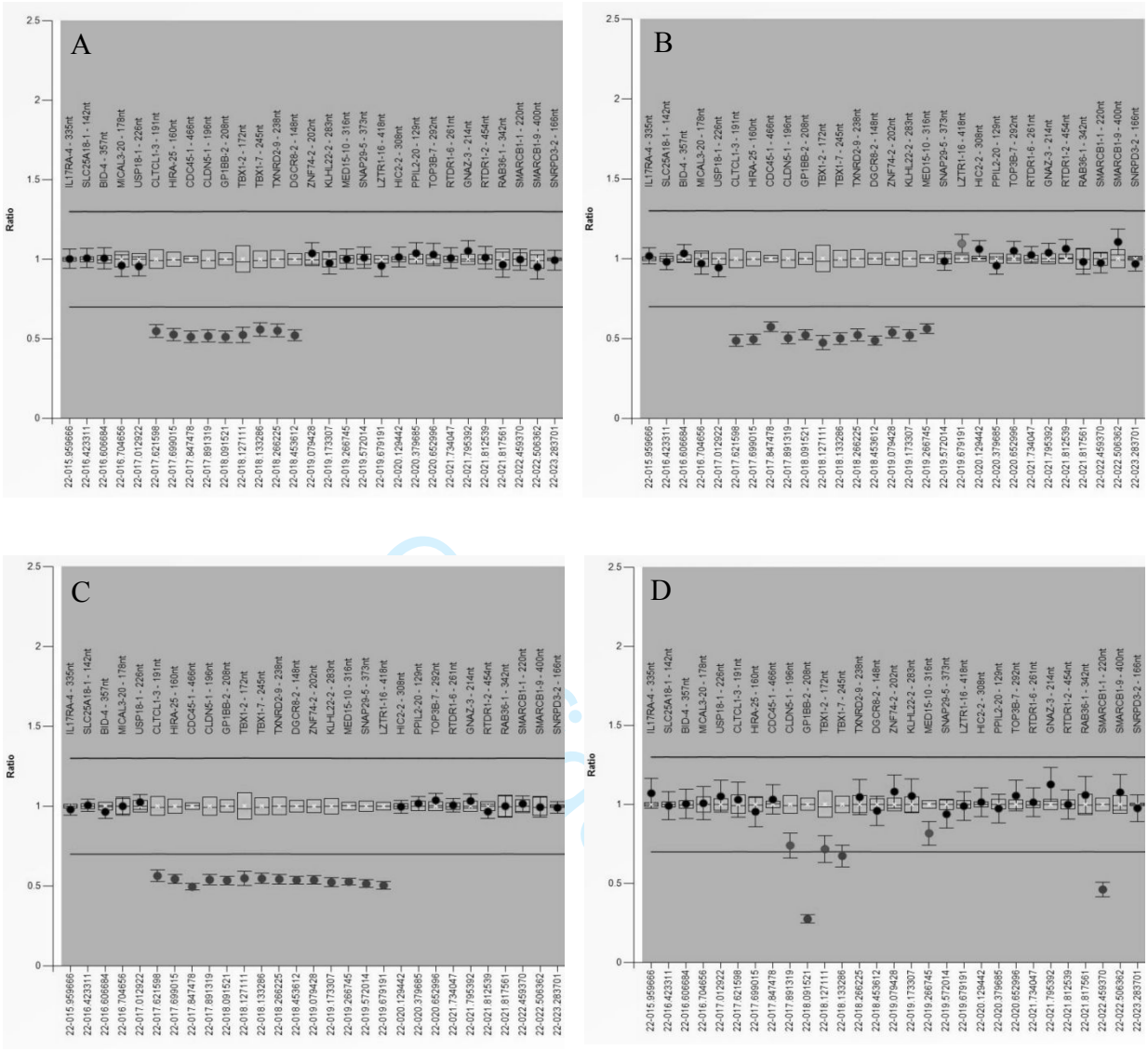

Figure S2. MLPA results for 22q11.2 deletions. The MLPA probes (MRC Holland) are listed at the top of the figure, from centomeric (left) to telomeric (right); for relative position of probes see Figure 1. The circles with error bars indicate the dosage quotient for each probe. The bottom horizontal line represents the cut-off point provided for a heterozygous 22q11.2 deletion.

A. Proximal nested LCR22A-LCR22B 22q11.2 deletion.

B. Proximal nested LCR22A-LCR22C 22q11.2 deletion.

C. Common LCR22A-LCR22D 22q11.2 deletion.

D. Small nested (atypical) 22q11.2 deletion (not meeting study criteria).

Supplemental References

1. Tomita-Mitchell, A. *et al.* Multiplexed quantitative real-time PCR to detect 22q11.2 deletion in patients with congenital heart disease. *Physiol. Genomics* **42A**, 52–60 (2010).
2. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif* **25**, 402–408 (2001).
3. DeLong, E. R., DeLong, D. M. & Clarke-Pearson, D. L. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* **44**, 837–845 (1988).
4. Kramer, M. S. *et al.* A new and improved population-based Canadian reference for birth weight for gestational age. *Pediatrics* **108**, E35 (2001).