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## A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Manuscripts

**Title****A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper**

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## Abstract

### Introduction:

The success rate of in vitro fertilisation (IVF) treatment for couples with infertility remains low due to lack of a reliable tool in selecting euploid embryos for transfer. This study aims to compare the efficacy in embryo selection based on morphology alone compared to non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation (IVF).

### Methods and analysis

This is a randomised double blind controlled trial conducted in two tertiary assisted reproduction centres. A total of 500 infertile women will be recruited and undergo IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into two groups: the intervention group using morphology and niPGT-A and the control group based on morphology alone. In the control group, blastocysts with the best quality morphology will be replaced first. In the intervention group, blastocysts with the best morphology and euploid result of spent culture medium will be replaced first. The primary outcome is a live birth per the first embryo transfer. The statistical analysis will be performed with the intention to treat and per protocol.

### Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the two participating units. All participants will provide written informed consent before joining the study. The results of the study will be submitted to scientific conferences and peer-reviewed journals.

### Registration details

The study was registered under Clinical Trials Registry (trial number NCT04474522).

### Strengths and limitations of this study

- This is a randomised double blind controlled clinical trial.
- There are no other similar studies registered.
- Our study is not designed to detect small differences in the live birth rates.

## Introduction

One in seven couples experience difficulty in conceiving. Many of them will require in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to stimulate a woman's ovaries to produce a number of oocytes which are collected by a minor operation and then mixed with sperm to form embryos in the laboratory. Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite advances in ovarian stimulation, culture medium and laboratory conditions, the pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2). The corresponding rates in the United States in 2016 were 45% and 36% per transfer respectively (<https://www.cdc.gov/art/reports/2016/national-summary.html>).

The success of IVF depends on selection of the most competent embryos for transfer, which is still based on morphological criteria by examining the appearance of the embryos under a microscope. But it is well known that many women fail to achieve a pregnancy even after transfer of what are perceived to be good quality embryos. Therefore, some clinics replace multiple embryos in order to maximise pregnancy rates, a strategy which is associated with a high risk of multiple pregnancy.

Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. It is a major reason for failure of pregnancy, miscarriage and congenital anomalies following both natural conception and IVF pregnancies and increases exponentially with maternal age (3-5).

Our inability to assess embryo quality and select those with the highest potential for implantation on the basis of morphology has led to the use of preimplantation genetic testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an embryo and assessment of the chromosome copy numbers. While PGT-A cannot create a healthy embryo or improve the quality of an embryo, it provides a method of selecting embryos with a normal number of chromosomes for transfer. This in turn has the potential to increase the chance of having a healthy live birth and reduce the risk of miscarriage or an abnormal fetus caused by an abnormal number of chromosomes.

Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing accuracy. A systematic review (6) of nine randomised controlled trials failed to demonstrate the benefit of the use of PGT-A with FISH.

Aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal i.e. comprehensive chromosome screening. Several small randomized controlled trials showed significantly higher pregnancy or live birth rate and lower miscarriage rate following the use of using chromosomal microarray analysis (7-9). The emergence of more advanced genome sequencing such as next generation sequencing (NGS) provides a reliable, high throughput

1  
2  
3 approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week,  
4 thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts  
5 will be frozen post biopsy and the blastocysts with normal genetic makeup will be  
6 thawed and replaced in a subsequent menstrual cycle. Cryopreservation of  
7 blastocysts and replacing the frozen blastocysts after thawing in subsequent cycles  
8 become a common practice with vitrification as the cryopreservation method (11).  
9  
10

11  
12 A systematic review (12) of the clinical utility of PGT-A with comprehensive  
13 chromosome screening found that three small randomised controlled trials  
14 demonstrated benefit in young and good prognosis patients in terms of clinical  
15 pregnancy rates and the use of single embryo transfer. However, a recent large  
16 randomised controlled trial (13) of 661 women comparing PGT-A using NGS vs  
17 morphology showed PGT-A did not improve overall pregnancy outcomes in all  
18 women aged 25–40 years with at least two blastocysts that could be biopsied. It is  
19 possible that there is a detrimental effect of the biopsy of blastocysts on the embryo  
20 viability that nullifies the benefit of PGT-A.  
21  
22

23  
24 The traditional PGT involves biopsy of a few cells from trophectoderm of a  
25 blastocyst, which requires skilful laboratory staff and additional instrumentation  
26 such as laser equipment. The trophectoderm biopsy is an invasive procedure and  
27 may lead to reduction in implantation potential, although the implantation potential  
28 is less affected when compared with blastomere biopsy from cleavage stage embryos  
29 (14). A non-invasive approach to PGT-A is definitely needed.  
30  
31

32  
33 The demonstration of release of cell-free DNA from human embryos into the  
34 surrounding environment (15) opens up the possibility of non-invasive PGT for  
35 aneuploidy (niPGT-A). Collection of spent culture medium (SCM) requires no  
36 specialized training and imposes negligible risk to the embryo. SCM may be more  
37 representative of the whole blastocyst as embryonic DNA is released from both  
38 trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains  
39 embryonic DNA from trophectoderm only. Multiple recent studies (16–23) have  
40 demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at  
41 the cleavage and blastocyst stages. It was shown that 24–48 hr of contact with the  
42 embryo was sufficient to collect cell-free DNA from SCM (21). The origin of cell-  
43 free DNA can be embryonic or parental. It is proposed that the cell-free DNA is  
44 derived from cells discarded by the embryos as a corrective mechanism for  
45 aneuploidies (16). However, the amount of cell-free DNA was not significantly  
46 greater in SCM from aneuploid versus euploid embryos, ruling out this possibility  
47 (20). Maternal and paternal contamination in SCM can be minimized by performing  
48 thorough oocyte stripping and intracytoplasmic sperm injection.  
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54  
55 In a recent review (24), the amplification success rate using sequential culture media  
56 for SCM ranged between 90% and 100% while the concordance rate in general  
57 ploidy i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high  
58 as 100% with an average of 75%. The difference in the general ploidy between SCM  
59 and trophectoderm biopsy can be due to mosaicism, which can be revealed in  
60

1  
2  
3 trophoctoderm biopsy but not in SCM due to the nature of the DNA source and  
4 relatively low embryonic DNA fraction.  
5  
6

7 All relevant studies on niPGT-A focus the amplification success and concordance  
8 rates between SCM and trophoctoderm biopsy. A large clinical randomised trial is  
9 urgently needed to confirm its efficacy in embryo selection during IVF in terms of  
10 live birth and miscarriage rates.  
11  
12

### 13 **Objective and hypothesis**

14 This randomised double blind controlled trial aims to compare the efficacy in  
15 embryo selection based on morphology alone compared to niPGT-A and  
16 morphology in infertile women undergoing IVF.  
17  
18

19 Hypothesis to be tested include:  
20

- 21 1. The embryo selection based on niPGT-A and morphology results in a higher  
22 live birth rate in IVF as compared with that based on morphology alone.
- 23 2. The embryo selection based on niPGT-A and morphology results in a lower  
24 miscarriage rate in IVF as compared with that based on morphology alone.  
25  
26

### 27 **Methods and analysis**

#### 28 **Trial design**

29 This is a randomised double blind controlled trial. Eligible women seeking fertility  
30 treatment in the Assisted Reproduction Units in Department of Obstetrics &  
31 Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited  
32 for the study and informed written consent will be obtained after counseling.  
33  
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35

36 Ethics approval was sought from the Institutional Review Board of the University  
37 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
38 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
39 KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry  
40 (Identifier NCT04474522). Written informed consent will be sought before joining  
41 the study.  
42  
43  
44

#### 45 **Selection and Withdrawal of subjects:**

46 The population for trial will be infertile women undergoing IVF.  
47  
48

#### 49 ***Inclusion criteria:***

50 Women admitted to the study will fulfil all of the following criteria:

- 51 • Age less than 43 years at the time of ovarian stimulation and
- 52 • Having at least two blastocysts suitable for freezing on day 6 after oocyte  
53 retrieval  
54  
55

#### 56 ***Exclusion criteria:***

57 Women should not be recruited in any of the following conditions:

- 58 • Having less than two blastocysts suitable for freezing on day 6 after oocyte  
59  
60

- 1  
2  
3 retrieval;
- 4 • Undergoing PGT for monogenic diseases or structural rearrangement of
  - 5 chromosomes;
  - 6
  - 7 • Using donor oocytes;
  - 8 • Having hydrosalpinx shown on pelvic scanning and not surgically treated
  - 9

### 10 ***Withdrawal criteria***

11 Participation in the study is totally voluntary. Recruited women can withdraw from  
12 the study at any time without giving any reasons and subsequently they will receive  
13 standard medical care.  
14  
15

### 16 **Treatment of subjects:**

#### 17 Methods

18 Eligible women will be recruited for the study and informed written consent will  
19 be obtained after counseling on the day of oocyte retrieval.  
20

#### 21 *IVF protocol*

22 Infertile women will undergo IVF as clinically indicated. They will receive ovarian  
23 stimulation as in standard operation procedure. Ovarian stimulation with  
24 gonadotropin injections (150-300 IU daily depending on the antral follicle count)  
25 will be given. Medroxyprogesterone acetate 10mg daily will be started on the day  
26 2 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be  
27 started on the day 6 of ovarian stimulation to prevent premature ovulation.  
28 Ultrasound monitoring will be performed to monitor the growth of follicles. When  
29 three follicles reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel  
30 0.25mg) or GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte  
31 retrieval will be scheduled 36 hours after the trigger under transvaginal ultrasound  
32 guidance.  
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38 Oocytes will be fertilized and normal fertilization will be assessed and confirmed  
39 by the presence of two pronuclei. Embryos will be grown individually to the  
40 blastocyst stage, usually day 5 or 6 after oocyte retrieval, in a monophasic medium.  
41 On Day 3, embryos will be rinsed briefly in fresh culture medium. The culture  
42 medium will be replenished and culture will be continued at 37°C and 6% CO<sub>2</sub> in  
43 reduced oxygen tension (5%). No fresh transfer of blastocysts will be performed  
44 in the stimulated cycle.  
45  
46  
47

#### 48 *Grading of blastocyst by morphology*

49 Blastocysts are graded according to Gardner's classification (25). Each blastocyst  
50 will be cryopreserved on day 6 by vitrification individually and its SCM (~8 µl)  
51 will be frozen at -80°C separately and individually. The embryologist will grade  
52 the morphology of blastocysts according to Gardner's criteria .  
53  
54  
55

56 Then, on day 6 after oocyte retrieval, women will then be randomly assigned by a  
57 PGT laboratory staff into one of the following two groups according to a computer-  
58 generated randomization list with a 1:1 ratio and a block size of 10. The  
59  
60



1  
2  
3 randomization list will be prepared by a research nurse, who is not involved in the  
4 clinical care of these women.

- 5 1. the intervention group using morphology and niPGT-A and
- 6 2. the control group based on morphology alone.

7  
8  
9 The women, clinicians and embryologists in the IVF laboratory will be blinded to  
10 the treatment groups they are assigned. Only the laboratory staff in the PGT  
11 laboratory will be aware of the group assignment.

#### 12 *niPGT-A of spent culture medium (SCM)*

13  
14 In the intervention group, comprehensive chromosome screening using NGS will  
15 be performed according to the recommendations of the company in all SCM  
16 samples. In the control group, the measurement will be done retrospectively after  
17 a live birth or when all blastocysts are replaced without a live birth.

18  
19 A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit,  
20 PerkinElmer) will be used to analyse the SCM samples. The protocol has been  
21 previously optimized with non-invasive samples from 15 laboratories around the  
22 world. The kit follows a single tube workflow, two-steps PCR to whole genome  
23 amplification of the DNA in SCM and then attaches indexes and sequence-specific  
24 adapters to template DNA, resulting in sequencing ready samples.

25  
26 After purification, equal molar concentration of indexed DNA from each sample  
27 will be pooled (96 samples) and then sequenced on a MiSeq system (Illumina) at  
28 1x75 bp read length. On-board secondary analysis will be performed automatically  
29 by the MiSeq Reporter (Illumina) followed by the PG-Find Software (v 1.0,  
30 PerkinElmer). Reads aligning to anomalous, unstructured and highly repetitive  
31 sequences will be filtered from the analysis. A target bin size of 1,000 kb will be  
32 used, giving a minimum resolution of 10 Mb. All genomic positions will be  
33 referred to the human genome build NCBI 37.

34  
35 According to the default setting of the PG-Find software, classification of  
36 aneuploidy is determined by CNV (copy number variation) value. CNV value >2.7  
37 is considered as gain while CNV value <1.3 is considered as loss. Sample will be  
38 concluded as non-euploid when one or more of the chromosomes show gain/loss.

39  
40 The niPGT-A result of the SCM sample can be euploid, non-euploid and non-  
41 informative. It will be used only to prioritize the sequence of embryo transfer.  
42 Blastocysts with non-euploid result in the niPGT-A report will not be discarded  
43 and will be transferred with lower priority.

#### 44 *Blinding*

45  
46 The embryologist will grade the morphology of blastocysts according to Gardner's  
47 criteria stated above and the grading of blastocysts will be entered into an online  
48 database, which will be managed by an IT technician. The laboratory staff in the  
49 PGT laboratory will enter the PGT result into an online database when the niPGT-  
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3 A results are available. The IT technician will merge the data online to compile the  
4 sequence of embryo transfer according to a pre-determined algorithm which  
5 depends on the day of blastocyst development (day 5 better than day 6), blastocyst  
6 morphology and niPGT-A result of the intervention group. The IT technician will  
7 issue the sequence of embryo transfer which does not contain information on the  
8 grading of the blastocyst and the NIPGT result to the embryologists in the IVF  
9 laboratory. Therefore, the subjects recruited, the clinicians and the embryologists  
10 will be blinded to the group allocation.  
11  
12  
13

#### 14 *A pilot study*

15 A pilot study was conducted on 82 SCM from February to September 2020. Media  
16 cultured in parallel but without contact with embryos were collected as controls  
17 (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and 72 SCM  
18 resulted in conclusive result (90.0%, 72/80). All controls showed no amplification.  
19  
20  
21

22 In this cohort, 40 SCM with conclusive results were collected from PGT cycles in  
23 which trophoctoderm biopsies were also performed. 85.0% (34/40) of samples  
24 showed concordance results between trophoctoderm biopsy and SCM.  
25  
26

#### 27 *Frozen embryo transfer (FET)*

28 Blastocysts will be replaced in the natural or hormonal replacement cycles,  
29 depending whether the women have regular menstrual cycles or not. Only one  
30 blastocyst will be transferred each time. The embryologists in the IVF laboratory  
31 will thaw and transfer the blastocyst according to the sequence of embryo transfer  
32 generated and issued by the IT technician. In the control group, blastocysts which  
33 develop on day 5 after the oocyte retrieval and have the best grading will be  
34 replaced first. In the intervention group, blastocysts which develop on day 5 and  
35 have the best grading and euploid result will be replaced first. If no blastocysts  
36 have euploid result, those with non-informative followed by non-euploid results  
37 will be replaced.  
38  
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41

#### 42 *Pregnancy*

43 A urine pregnancy test will be performed 14 days after the transfer. If the  
44 pregnancy test is positive, transvaginal ultrasound will be performed two weeks  
45 later to locate the pregnancy and confirm foetal viability and the number of fetuses.  
46 Subsequent management will be the same as other women with early pregnancy.  
47 They will be referred for antenatal care when the ongoing pregnancy is 8-10 weeks.  
48  
49  
50

#### 51 *Follow-up*

52 Written consent regarding retrieval of pregnancy and delivery data will be sought  
53 from the recruited women at the time of study. The women will be contacted after  
54 delivery by phone to retrieve the information of the pregnancy outcomes. The  
55 outcome of the pregnancy (delivery, miscarriage), number of babies born, birth  
56 weights and obstetrics complications will be recorded.  
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3 Women in both groups will continue to have blastocyst transfer until all the  
4 cryopreserved blastocysts are used up or they become pregnant within 6 months  
5 after randomisation. Cumulative live birth rate will be calculated (the number of  
6 live birth per couple within the study period). The pregnancy complication and  
7 congenital abnormalities of the pregnancies in the two groups will be traced  
8 through hospital records or patient contact by mail or phone of mail and compared.  
9  
10

### 11 **Assessment of outcomes:**

12 The primary outcome is live birth beyond 22 weeks of gestation per the first FET.  
13  
14

15 Secondary outcomes include

- 16 • Cumulative live birth rate: the number of pregnancies leading to live birth within  
17 6 months of randomization.
- 18 • Time to pregnancy
- 19 • Positive urine pregnancy test
- 20 • Clinical pregnancy defined as presence of intrauterine gestational sac on  
21 scanning at gestational week 6.
- 22 • Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of  
23 gestation
- 24 • Miscarriage defined as a clinically recognized pregnancy loss before the 22  
25 weeks of pregnancy and whose denominator is the clinical pregnancy.
- 26 • Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of  
27 gestation
- 28 • Ectopic pregnancy
- 29 • Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational  
30 diabetes, congenital anomaly, perinatal mortality and birthweight of newborn.  
31

32 Consistency of case management:

33 The same standardised study protocol will be adopted in the two study centres. The  
34 clinicians who manage the women in KWH had all been trained at QMH, and are  
35 now adopting the same clinical management protocols in their respective unit.  
36 Regular and frequent communication among the two participating centers will also  
37 ensure dissemination of updated information on recruitment and safety issues.  
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45 Patient and Public involvement

46 Patients or the public were not involved in the design, conduct, reporting or  
47 dissemination plans of this study.  
48  
49

### 50 **Statistics**

#### 51 ***Sample size calculation***

52 From the data in QMH and KWH, the live birth rate following transfer of one  
53 blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate  
54 following blastocyst morphology and NIPGT-A, the live birth rate will increase from  
55 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate  
56 observed following conventional PGT-A in the centre. The calculated sample size is  
57 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming  
58 a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group.  
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4 A pre-specified subgroup analysis will be performed: women aged <35 years vs  
5 >=35 years.  
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### 8 **Data analysis**

9 Demographic features of women in the two groups will be compared. Comparison  
10 of quantitative variables will be performed using Student's t, while categorical  
11 variables will be compared using a Chi-square analysis. All statistical analyses of  
12 the data will be performed with the intention to treat and per protocol using the SPSS  
13 program V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be  
14 considered statistically significant.  
15  
16

17  
18 The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will  
19 be calculated.  
20  
21

### 22 **Ethics and dissemination**

23 Ethics approval was sought from the Institutional Review Board of the University  
24 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
25 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
26 KC/KE-20-0098/FR-2). All participants will provide written informed consent  
27 before randomization. The research findings from this study will be submitted to  
28 scientific conferences and peer-reviewed journals for publication so as to  
29 disseminate the results to other researchers and clinicians working in the field.  
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32

### 33 **Discussion**

34 The success rate of IVF has long been limited due to the inability to assess the  
35 implantation potential of each embryo accurately. Assessment by morphology using  
36 Gardner's classification could not reflect chromosomal abnormalities in embryos,  
37 which is the reason for failure of implantation in most of the cases. NGS offers  
38 several advantages over chromosomal microarray analysis by (i) reduced DNA  
39 sequencing cost by high throughput sequencing technologies and a high number of  
40 samples can be simultaneously sequenced in a single testing; (ii) enhanced detection  
41 of partial or segmental aneuploidies as a result of the increase in chromosomal  
42 analysis resolution to a few mega bases; (iii) increased dynamic range enabling  
43 enhanced detection of mosaicism in multicellular samples and (iv) automation of the  
44 sequencing library preparation and automation of the PGT-A diagnostic procedure.  
45 However, the beneficial effect of PGT-A has been nullified by the detrimental effect  
46 of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst  
47 evaluation tool than the traditional morphology assessment during IVF treatment, it  
48 can potentially shorten the time-to-pregnancy in women with infertility.  
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55 Currently, all relevant studies on niPGT-A focused on the amplification success and  
56 concordance rates between SCM and trophectoderm biopsy but not on assessing its  
57 ability as a screening tool for blastocyst transfer prioritization. This study could  
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3 provide valuable information on the potential novel use of niPGT-A as an adjunct  
4 for morphological assessment of blastocysts.  
5  
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7

### 8 **Trial status**

9 The first subject was recruited on 1<sup>st</sup> July 2021 and 400 women were recruited up to  
10 the writing of this protocol paper.  
11  
12

### 13 **Full references:**

- 14 1. NICE. Fertility: assessment and treatment for people with fertility  
15 problems. NICE guideline CG11. 2014.
- 16 2. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko  
17 T, Scaravelli G, Smeenk J, Vidakovic S, Goossens V; European IVF-  
18 monitoring Consortium (EIM) for the European Society of Human  
19 Reproduction and Embryology (ESHRE). ART in Europe, 2014: results  
20 generated from European registries by ESHRE: The European IVF-  
21 monitoring Consortium (EIM) for the European Society of Human  
22 Reproduction and Embryology (ESHRE). *Hum Reprod*. 2018 Sep  
23 1;33(9):1586-1601.
- 24 3. Spandorfer SD, Davis OK, Barmat LI, Chung PH, Rosenwaks Z.  
25 Relationship between maternal age and aneuploidy in in vitro fertilization  
26 pregnancy loss. *Fertility and sterility*. 2004;81(5):1265-9.
- 27 4. Hassold T, Hall H, Hunt P. The origin of human aneuploidy: where we have  
28 been, where we are going. *Human molecular genetics*. 2007;16(R2):R203-  
29 R8.
- 30 5. Andersen A-MN, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal  
31 age and fetal loss: population based register linkage study. *BMJ*.  
32 2000;320(7251):1708-12.
- 33 6. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation  
34 genetic screening: a systematic review and meta-analysis of RCTs. *Hum*  
35 *Reprod Update*. 2011 Jul-Aug;17(4):454-66.
- 36 7. Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of  
37 single blastocysts for fresh transfer via standard morphology assessment  
38 alone and with array CGH for good prognosis IVF patients: results from a  
39 randomized pilot study. *Molecular cytogenetics*. 2012;5(1):1-8.
- 40 8. Scott Jr RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al.  
41 Blastocyst biopsy with comprehensive chromosome screening and fresh  
42 embryo transfer significantly increases in vitro fertilization implantation  
43 and delivery rates: a randomized controlled trial. *Fertility and Sterility*.  
44 2013;100(3):697-703.
- 45 9. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, et al. In vitro  
46 fertilization with single euploid blastocyst transfer: a randomized controlled  
47 trial. *Fertility and Sterility*. 2013;100(1):100-7. e1.
- 48 10. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et  
49 al. Development and validation of a next-generation sequencing-based  
50 protocol for 24-chromosome aneuploidy screening of embryos. *Fertility*  
51  
52  
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- and Sterility. 2014;101(5):1375-82.
11. Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T, Salamonsen LA, Rombauts L. Fresh versus frozen embryo transfer: backing clinical decisions with scientific and clinical evidence. *Human Reproduction Update*. 2014 Nov-Dec;20(6):808-21.
  12. Lee E, Illingworth P, Wilton L, Chambers GM. The clinical effectiveness of preimplantation genetic diagnosis for aneuploidy in all 24 chromosomes (PGD-A): systematic review. *Hum Reprod*. 2015 Feb;30(2):473-83.
  13. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M, Wells D, Gordon T, Stock-Myer S, Willman S; STAR Study Group. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertility and Sterility*. 2019 Dec;112(6):1071-1079.
  14. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertility and Sterility*. 2013;100(3):624-30.
  15. Assou S, Aït-Ahmed O, El Messaoudi S, Thierry AR, Hamamah S. Noninvasive pre-implantation genetic diagnosis of X-linked disorders. *Med Hypotheses* 2014;83:506–508.
  16. Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertil Steril*. 2017;107(1):220–8.
  17. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci USA*. 2016;113(42):11907–12.
  18. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril*. 2016;106(6):1312–8.
  19. Feichtinger M, Vaccari E, Carli L, Wallner E, Mädler U, Figl K, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of concept pilot study. *Reprod BioMed Online*. 2017;34(6):583–9.
  20. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod*. 2018;33(4):745–56.
  21. Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, Ibarrientos Z, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. *PLoS One*. 2018;13(5): e0197262.
  22. Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, et al.

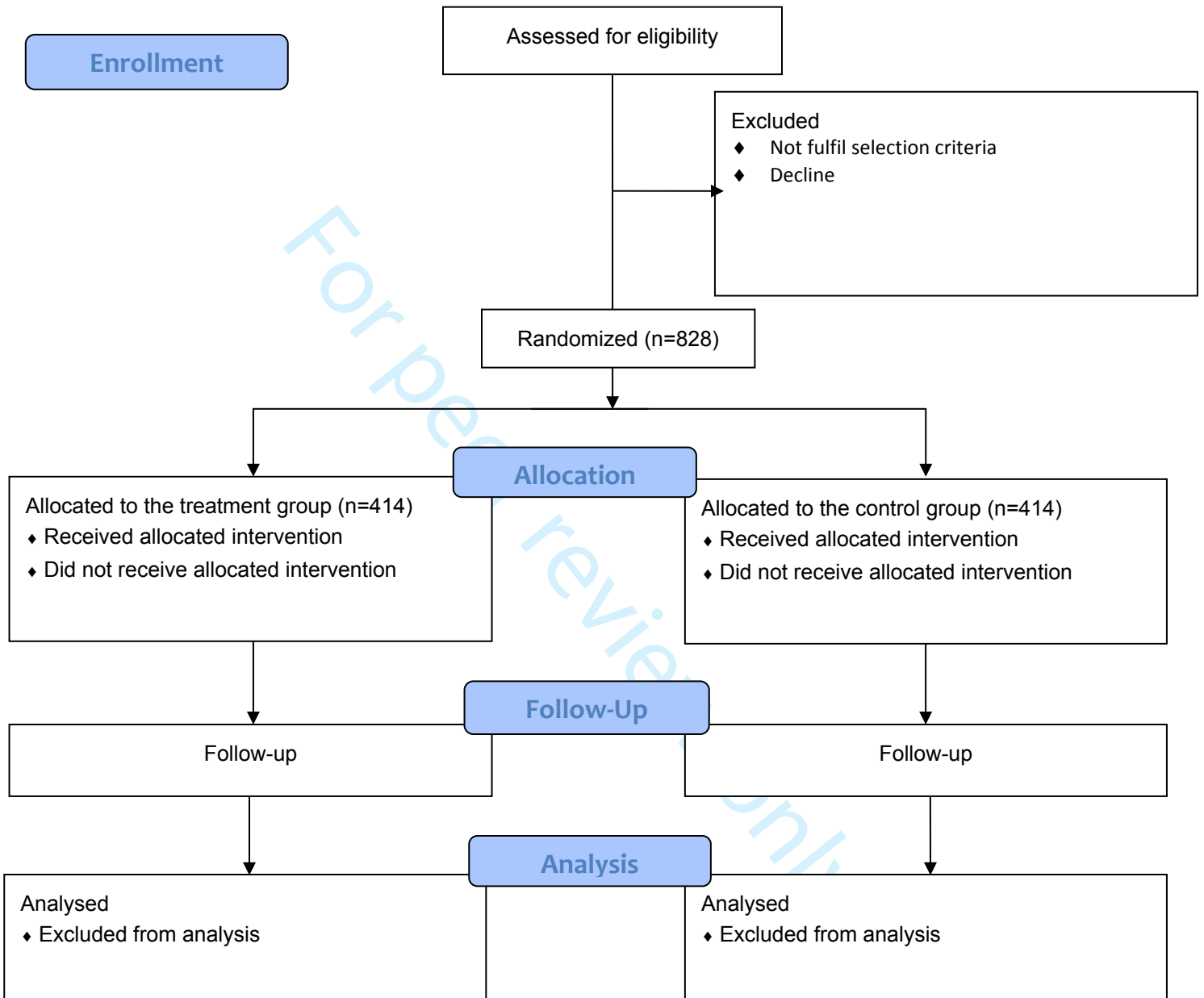
- 1  
2  
3 Pushing the limits of detection: investigation of cell-free DNA for  
4 aneuploidy screening in embryos. *Fertil Steril*. 2018;110(3): 467–75.  
5  
6 23. Capalbo A, Romanelli V, Patassini C, Poli M, Girardi L, Giancani A, et al.  
7 Diagnostic efficacy of blastocoel fluid and spent media as sources of DNA  
8 for preimplantation genetic testing in standard clinical conditions. *Fertil*  
9 *Steril*. 2018;110(5):870–9.  
10  
11 24. Belandres D, Shamonki M, Arrach N. Current status of spent embryo media  
12 research for preimplantation genetic testing. *J Assist Reprod Genet*. 2019  
13 May;36(5):819-826.  
14  
15 25. Gardner D, Schoolcraft W, Jansen R, Mortimer D. Towards reproductive  
16 certainty: infertility and genetics beyond. *vitro culture of human*  
17 *blastocysts*, Carnforth, Parthenon Press, pp378. 1999;388.  
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### CONSORT 2010 Flow Diagram





# BMJ Open

## A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Keywords:	Subfertility < GYNAECOLOGY, Reproductive medicine < GYNAECOLOGY, GENETICS

SCHOLARONE™  
Manuscripts

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3 1 **Title**

4 2 **A randomised double blind controlled trial of non-invasive preimplantation**  
5 3 **genetic testing for aneuploidy in in vitro fertilisation: a protocol paper**

6 4  
7 5 Heidi H.Y. Cheng<sup>1</sup>, Judy F.C. Chow<sup>1</sup>, Kevin K.W. Lam<sup>1</sup>, Shui Fan Lai <sup>1,2</sup>, William  
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## 21 **Abstract**

### 22 **Introduction:**

23 The success rate of in vitro fertilisation (IVF) treatment for couples with infertility  
24 remains low due to lack of a reliable tool in selecting euploid embryos for transfer.  
25 This study aims to compare the efficacy in embryo selection based on morphology  
26 alone compared to non-invasive preimplantation genetic testing for aneuploidy  
27 (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation  
28 (IVF).  
29

### 30 **Methods and analysis**

31 This is a randomised double blind controlled trial conducted in two tertiary assisted  
32 reproduction centres. A total of 500 infertile women will be recruited and undergo  
33 IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into  
34 two groups: the intervention group using morphology and niPGT-A and the control  
35 group based on morphology alone. In the control group, blastocysts with the best  
36 quality morphology will be replaced first. In the intervention group, blastocysts with  
37 the best morphology and euploid result of spent culture medium will be replaced  
38 first. The primary outcome is a live birth per the first embryo transfer. The statistical  
39 analysis will be performed with the intention to treat and per protocol.  
40

### 41 **Ethics and dissemination**

42 Ethics approval was sought from the Institutional Review Board of the two  
43 participating units. All participants will provide written informed consent before  
44 joining the study. The results of the study will be submitted to scientific conferences  
45 and peer-reviewed journals.  
46

### 47 **Registration details**

48 The study was registered under ClinicalTrials.gov (trial number NCT04474522).  
49  
50

### 51 **Strengths and limitations of this study**

- 52 • This is a randomised double blind controlled clinical trial.
- 53 • The recruitment takes place in a large representative stakeholder group from  
54 the field of reproductive medicine in Hong Kong.
- 55 • The intervention group of this study includes both morphology and niPGT-A,  
56 thereby investigating the sole effect of addition of niPGT-A on embryo  
57 prioritization alongside with morphology assessment
- 58 • One limitation is that our study is not designed to detect small differences in  
59 the live birth rates.  
60

## 61 Introduction

62 One in seven couples experience difficulty in conceiving. Many of them will require  
63 in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to  
64 stimulate a woman's ovaries to produce a number of oocytes which are collected by  
65 a minor operation and then mixed with sperm to form embryos in the laboratory.  
66 Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage  
67 embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite  
68 advances in ovarian stimulation, culture medium and laboratory conditions, the  
69 pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2).  
70 The corresponding rates in the United States in 2016 were 45% and 36% per transfer  
71 respectively (<https://www.cdc.gov/art/reports/2016/national-summary.html>).

72  
73 The success of IVF depends on selection of the most competent embryos for transfer,  
74 which is still based on morphological criteria by examining the appearance of the  
75 embryos under a microscope. But it is well known that many women fail to achieve  
76 a pregnancy even after transfer of what are perceived to be good quality embryos.  
77 Therefore, some clinics replace multiple embryos in order to maximise pregnancy  
78 rates, a strategy which is associated with a high risk of multiple pregnancy.

79  
80 Chromosome aneuploidy is an error in cell division that results in the "daughter"  
81 cells having the wrong number of chromosomes. In some cases there is a missing  
82 chromosome, while in others an extra one. It is a major reason for failure of  
83 pregnancy, miscarriage and congenital anomalies following both natural conception  
84 and IVF pregnancies and increases exponentially with maternal age (3-5).

85  
86 Our inability to assess embryo quality and select those with the highest potential for  
87 implantation on the basis of morphology has led to the use of preimplantation genetic  
88 testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an  
89 embryo and assessment of the chromosome copy numbers. While PGT-A cannot  
90 create a healthy embryo or improve the quality of an embryo, it provides a method  
91 of selecting embryos with a normal number of chromosomes for transfer. This in  
92 turn has the potential to increase the chance of having a healthy live birth and reduce  
93 the risk of miscarriage or an abnormal fetus caused by an abnormal number of  
94 chromosomes.

95  
96 Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can  
97 only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing  
98 accuracy. A systematic review of nine randomised controlled trials failed to  
99 demonstrate the benefit of the use of PGT-A with FISH(6).

100  
101 Aneuploidy screening of all chromosomes is necessary to determine whether an  
102 embryo is chromosomally normal i.e. comprehensive chromosome screening.  
103 Several small randomized controlled trials showed significantly higher pregnancy or  
104 live birth rate and lower miscarriage rate following the use of using chromosomal  
105 microarray analysis (7-9). The emergence of more advanced genome sequencing  
106 such as next generation sequencing (NGS) provides a reliable, high throughput

1  
2  
3 107 approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week,  
4 108 thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts  
5 109 are frozen post biopsy and the blastocysts with normal genetic makeup are thawed  
6 110 and replaced in a subsequent menstrual cycle. Cryopreservation of blastocysts and  
7 111 replacing the frozen blastocysts after thawing in subsequent cycles become a  
8 112 common practice with vitrification as the cryopreservation method (11).  
9 113

12 114 A systematic review of the clinical utility of PGT-A with comprehensive  
13 115 chromosome screening found that three small randomised controlled trials  
14 116 demonstrated benefit in young and good prognosis patients in terms of clinical  
15 117 pregnancy rates and the use of single embryo transfer (12). However, a recent large  
16 118 randomised controlled trial of 661 women comparing PGT-A using NGS vs  
17 119 morphology showed PGT-A did not improve overall pregnancy outcomes in all  
18 120 women aged 25–40 years with at least two blastocysts that could be biopsied (13).  
19 121 It is possible that there is a detrimental effect of the biopsy of blastocysts on the  
20 122 embryo viability that nullifies the benefit of PGT-A.  
21 123

24 124 The traditional PGT involves biopsy of a few cells from trophectoderm of a  
25 125 blastocyst, which requires skilful laboratory staff and additional instrumentation  
26 126 such as laser equipment. The trophectoderm biopsy is an invasive procedure and  
27 127 may lead to reduction in implantation potential, although the implantation potential  
28 128 is less affected when compared with blastomere biopsy from cleavage stage embryos  
29 129 (14). A non-invasive approach to PGT-A is definitely needed.  
30 130

33 131 The demonstration of release of cell-free DNA from human embryos into the  
34 132 surrounding environment opens up the possibility of non-invasive PGT for  
35 133 aneuploidy (niPGT-A)(15). Collection of spent culture medium (SCM) requires no  
36 134 specialized training and imposes negligible risk to the embryo. SCM may be more  
37 135 representative of the whole blastocyst as embryonic DNA is released from both  
38 136 trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains  
39 137 embryonic DNA from trophectoderm only. Multiple recent studies have  
40 138 demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at  
41 139 the cleavage and blastocyst stages(16-23). It was shown that 24–48 hr of contact  
42 140 with the embryo was sufficient to collect cell-free DNA from SCM (21). The origin  
43 141 of cell-free DNA can be embryonic or parental. It is proposed that the cell-free DNA  
44 142 is derived from cells discarded by the embryos as a corrective mechanism for  
45 143 aneuploidies (16,24). However, the amount of cell-free DNA was not significantly  
46 144 greater in SCM from aneuploid versus euploid embryos, ruling out this possibility  
47 145 (20). Maternal and paternal contamination in SCM can be minimized by performing  
48 146 thorough oocyte stripping and intracytoplasmic sperm injection(25).  
49 147

52 148 In a recent review, the amplification success rate using sequential culture media for  
53 149 SCM ranged between 90% and 100% while the concordance rate in general ploidy  
54 150 i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high as  
55 151 100% with an average of 75% (26). The difference in the general ploidy between  
56 152 SCM and trophectoderm biopsy can be due to mosaicism, which can be revealed in

1  
2  
3 153 trophoctoderm biopsy but not in SCM due to the nature of the DNA source and  
4 154 relatively low embryonic DNA fraction.

5 155  
6 156 All relevant studies on niPGT-A focus the amplification success and concordance  
7 157 rates between SCM and trophoctoderm biopsy. A large clinical randomised trial is  
8 158 urgently needed to confirm its efficacy in embryo selection during IVF in terms of  
9 159 live birth and miscarriage rates.

10 160

### 11 161 **Objective and hypothesis**

12 162 This randomised double blind controlled trial aims to compare the efficacy in  
13 163 embryo selection based on morphology alone compared to niPGT-A and  
14 164 morphology in infertile women undergoing IVF.

15 165

16 166 Hypothesis to be tested include:

- 17 167 1. The embryo selection based on niPGT-A and morphology results in a higher  
18 168 live birth rate in IVF as compared with that based on morphology alone.  
19 169 2. The embryo selection based on niPGT-A and morphology results in a lower  
20 170 miscarriage rate in IVF as compared with that based on morphology alone.

21 171

### 22 172 **Methods and analysis**

#### 23 173 **Trial design**

24 174 This is a randomised double blind controlled trial. Eligible women seeking fertility  
25 175 treatment in the Assisted Reproduction Units in Department of Obstetrics &  
26 176 Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited  
27 177 for the study and informed written consent will be obtained after counseling.

28 178

29 179 Ethics approval was sought from the Institutional Review Board of the University  
30 180 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
31 181 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
32 182 KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry  
33 183 (Identifier NCT04474522). Written informed consent will be sought before joining  
34 184 the study.

35 185

#### 36 186 **Selection and Withdrawal of subjects:**

37 187 The population for trial will be infertile women undergoing IVF.

38 188

#### 39 189 ***Inclusion criteria:***

40 190 Women admitted to the study will fulfil all of the following criteria:

- 41 191 • Age less than 43 years at the time of ovarian stimulation and  
42 192 • Having at least two blastocysts suitable for freezing on day 6 after oocyte  
43 193 retrieval

44 194

#### 45 195 ***Exclusion criteria:***

46 196 Women should not be recruited in any of the following conditions:

- 47 197 • Having less than two blastocysts suitable for freezing on day 6 after oocyte  
48 198

- 198 retrieval;
- 199 • Undergoing PGT for monogenic diseases or structural rearrangement of
- 200 chromosomes;
- 201 • Using donor oocytes;
- 202 • Having hydrosalpinx shown on pelvic scanning and not surgically treated

### 203

### 204 ***Withdrawal criteria***

205 Participation in the study is totally voluntary. Recruited women can withdraw from

206 the study at any time without giving any reasons and subsequently they will receive

207 standard medical care.

### 208

### 209 **Treatment of subjects:**

#### 210 Methods

211 Eligible women will be recruited for the study and informed written consent

212 will be obtained after counseling on the day of oocyte retrieval by research

213 nurse or clinicians.

#### 214

#### 215 *IVF protocol*

216 Infertile women will undergo IVF as clinically indicated. They will receive ovarian

217 stimulation as in standard operation procedure. Ovarian stimulation with

218 gonadotropin injections (150-300 IU daily depending on the antral follicle count)

219 will be given. Medroxyprogesterone acetate 10mg daily will be started on the day 2

220 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be started

221 on the day 6 of ovarian stimulation to prevent premature ovulation. Ultrasound

222 monitoring will be performed to monitor the growth of follicles. When three follicles

223 reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel 0.25mg) or

224 GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte retrieval will be

225 scheduled 36 hours after the trigger under transvaginal ultrasound guidance.

226

227 Oocytes will be fertilized conventionally or by intracytoplasmic sperm

228 injection (ICSI) depending on the semen parameters in accordance with the

229 standard operating procedures and normal fertilization will be assessed and

230 confirmed by the presence of two pronuclei. Embryos will be grown

231 individually to the blastocyst stage, up to day 6 after oocyte retrieval, in a

232 monophasic medium. On Day 3, embryos will be rinsed briefly in fresh culture

233 medium. The culture medium will be replenished and culture will be continued

234 at 37°C and 6% CO<sub>2</sub> in reduced oxygen tension (5%). No fresh transfer of

235 blastocysts will be performed in the stimulated cycle.

#### 236

#### 237 *Grading of blastocyst by morphology*

238 Blastocysts are graded according to Gardner's classification (27). Each

239 blastocyst will be cryopreserved on day 6 by vitrification individually and its

240 SCM (~8 µl) will be frozen at -80°C separately and individually. The

241 embryologist will grade the morphology of blastocysts according to Gardner's

242 criteria .

243

244 Then, on day 6 after oocyte retrieval, women will then be randomly assigned  
245 by a PGT laboratory staff into one of the following two groups according to a  
246 computer-generated randomization list with a 1:1 ratio and a block size of 10.  
247 The randomization list will be prepared by a research nurse, who is not  
248 involved in the clinical care of these women.

- 249 1. the intervention group using morphology and niPGT-A and
- 250 2. the control group based on morphology alone.

251

252 The women, clinicians and embryologists in the IVF laboratory will be blinded  
253 to the treatment groups they are assigned. Only the laboratory staff in the PGT  
254 laboratory will be aware of the group assignment.

255

#### 256 *niPGT-A of spent culture medium (SCM)*

257 In the intervention group, comprehensive chromosome screening using NGS  
258 will be performed according to the recommendations of the company in all  
259 SCM samples. In the control group, the measurement will be done  
260 retrospectively after a live birth or when all blastocysts are replaced without a  
261 live birth.

262

263 A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit,  
264 PerkinElmer) will be used to analyse the SCM samples. The protocol has been  
265 previously optimized with non-invasive samples from 15 laboratories around  
266 the world. The kit follows a single tube workflow, two-steps PCR to whole  
267 genome amplification of the DNA in SCM and then attaches indexes and  
268 sequence-specific adapters to template DNA, resulting in sequencing ready  
269 samples.

270

271 After purification, equal molar concentration of indexed DNA from each  
272 sample will be pooled (96 samples) and then sequenced on a MiSeq system  
273 (Illumina) at 1x75 bp read length. On-board secondary analysis will be  
274 performed automatically by the MiSeq Reporter (Illumina) followed by the  
275 PG-Find Software (v 1.0, PerkinElmer). Reads aligning to anomalous,  
276 unstructured and highly repetitive sequences will be filtered from the analysis.  
277 A target bin size of 1,000 kb will be used, giving a minimum resolution of 10  
278 Mb. All genomic positions will be referred to the human genome build NCBI  
279 37.

280

281 According to the default setting of the PG-Find software, classification of  
282 aneuploidy is determined by CNV (copy number variation) value. CNV value  
283  $>2.7$  is considered as gain while CNV value  $<1.3$  is considered as loss. Sample  
284 will be concluded as non-euploid when one or more of the chromosomes show  
285 gain/loss.

286

287 The niPGT-A result of the SCM sample can be euploid, non-euploid and non-  
288 informative. It will be used only to prioritize the sequence of embryo transfer.



1  
2  
3 289 Blastocysts with non-euploid result in the niPGT-A report will not be discarded and  
4 290 will be transferred with lower priority.  
5

6 291

### 7 292 *Blinding*

8 293 The embryologist will grade the morphology of blastocysts according to  
9 294 Gardner's criteria stated above and the grading of blastocysts will be entered  
10 295 into an online database, which will be managed by an IT technician. The  
11 296 laboratory staff in the PGT laboratory will enter the PGT result into an online  
12 297 database when the niPGT-A results are available. The IT technician will merge  
13 298 the data online to compile the sequence of embryo transfer according to a pre-  
14 299 determined algorithm which depends on the day of blastocyst development  
15 300 (day 5 better than day 6), blastocyst morphology and niPGT-A result of the  
16 301 intervention group. The IT technician will issue the sequence of embryo  
17 302 transfer which does not contain information on the grading of the blastocyst  
18 303 and the NIPGT result to the embryologists in the IVF laboratory. Therefore,  
19 304 the subjects recruited, the clinicians and the embryologists will be blinded to  
20 305 the group allocation.  
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26 306

### 27 307 *A pilot study*

28 308 A pilot study was conducted on 82 SCM from February to September 2020.  
29 309 Media cultured in parallel but without contact with embryos were collected as  
30 310 controls (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and  
31 311 72 SCM resulted in conclusive result (90.0%, 72/80). All controls showed no  
32 312 amplification.  
33

34 313

35 314 In this cohort, 40 SCM with conclusive results were collected from PGT cycles  
36 315 in which trophoctoderm biopsies were also performed. 85.0% (34/40) of  
37 316 samples showed concordance results between trophoctoderm biopsy and  
38 317 SCM.  
39

40 318

### 41 319 *Frozen embryo transfer (FET)*

42 320 Blastocysts will be replaced in the natural or hormonal replacement cycles,  
43 321 depending whether the women have regular menstrual cycles or not. Only one  
44 322 blastocyst will be transferred each time. The embryologists in the IVF laboratory  
45 323 will thaw and transfer the blastocyst according to the sequence of embryo transfer  
46 324 generated and issued by the IT technician. In the control group, blastocysts which  
47 325 develop on day 5 after the oocyte retrieval and have the best grading will be replaced  
48 326 first. In the intervention group, blastocysts which develop on day 5 and have the best  
49 327 grading and euploid result will be replaced first. If no blastocysts have euploid result,  
50 328 those with non-informative followed by non-euploid results will be replaced.  
51

52 329

### 53 330 *Pregnancy*

54 331 A urine pregnancy test will be performed 14 days after the transfer. If the  
55 332 pregnancy test is positive, transvaginal ultrasound will be performed two  
56 333 weeks later to locate the pregnancy and confirm foetal viability and the number  
57 334 of fetuses. Subsequent management will be the same as other women with

335 early pregnancy. They will be referred for antenatal care when the ongoing  
336 pregnancy is 8-10 weeks.

337

### 338 *Follow-up*

339 Written consent regarding retrieval of pregnancy and delivery data will be sought  
340 from the recruited women at the time of study. The women will be contacted after  
341 delivery by phone to retrieve the information of the pregnancy outcomes. The  
342 outcome of the pregnancy (delivery, miscarriage), number of babies born, birth  
343 weights and obstetrics complications will be recorded.

344

345 Women in both groups will continue to have blastocyst transfer until all the  
346 cryopreserved blastocysts are used up or they become pregnant within 6 months after  
347 randomisation. Cumulative live birth rate will be calculated (the number of live birth  
348 per couple within the study period). The pregnancy complication and congenital  
349 abnormalities of the pregnancies in the two groups will be traced through hospital  
350 records or patient contact by mail or phone of mail and compared.

351

### 352 **Assessment of outcomes:**

353 The primary outcome is live birth beyond 22 weeks of gestation per the first FET.

354

355 Secondary outcomes include

- 356 • Cumulative live birth rate: the number of pregnancies leading to live birth within  
357 6 months of randomization.
- 358 • Time to pregnancy
- 359 • Positive urine pregnancy test
- 360 • Clinical pregnancy defined as presence of intrauterine gestational sac on  
361 scanning at gestational week 6.
- 362 • Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of  
363 gestation
- 364 • Miscarriage defined as a clinically recognized pregnancy loss before the 22  
365 weeks of pregnancy and whose denominator is the clinical pregnancy.
- 366 • Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of  
367 gestation
- 368 • Ectopic pregnancy
- 369 • Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational  
370 diabetes, congenital anomaly, perinatal mortality and birthweight of newborn.

371

372 Consistency of case management:

373 The same standardised study protocol will be adopted in the two study centres. The  
374 clinicians who manage the women in KWH had all been trained at QMH, and are  
375 now adopting the same clinical management protocols in their respective unit.  
376 Regular and frequent communication among the two participating centers will also  
377 ensure dissemination of updated information on recruitment and safety issues.

378

379 *Patient and Public involvement*

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3 380 Patients or the public were not involved in the design, conduct, reporting or  
4 381 dissemination plans of this study.  
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## 7 383 **Statistics**

### 8 384 *Sample size calculation*

9  
10 385 From the data in QMH and KWH, the live birth rate following transfer of one  
11 386 blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate  
12 387 following blastocyst morphology and NIPGT-A, the live birth rate will increase from  
13 388 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate  
14 389 observed following conventional PGT-A in the centre. The calculated sample size is  
15 390 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming  
16 391 a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group.  
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20 393 A pre-specified subgroup analysis will be performed: women aged <35 years vs  
21 394 >=35 years.  
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23 395

### 24 396 *Data analysis*

25 397 Demographic features of women in the two groups will be compared. Comparison  
26 398 of quantitative variables will be performed using Student's t or Mann-whitney U-  
27 399 test where appropriate, while categorical variables will be compared using a Chi-  
28 400 square analysis, multivariable logistic regression or the one-way ANOVA test if  
29 401 more than two categories will be compared. All statistical analyses of the data will be  
30 402 performed with the intention to treat and per protocol using the SPSS program  
31 403 V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be considered  
32 404 statistically significant.  
33  
34 405

35  
36 406 The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will  
37 407 be calculated.  
38  
39 408

### 40 409 **Ethics and dissemination**

41 410 Ethics approval was sought from the Institutional Review Board of the University  
42 411 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
43 412 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
44 413 KC/KE-20-0098/FR-2). All participants will provide written informed consent  
45 414 before randomization. The research findings from this study will be submitted to  
46 415 scientific conferences and peer-reviewed journals for publication so as to  
47 416 disseminate the results to other researchers and clinicians working in the field.  
48  
49 417

### 50 418 **Discussion**

51 419 The success rate of IVF has long been limited due to the inability to assess the  
52 420 implantation potential of each embryo accurately. Assessment by morphology using  
53 421 Gardner's classification could not reflect chromosomal abnormalities in embryos,  
54 422 which is the reason for failure of implantation in most of the cases. NGS offers  
55 423 several advantages over chromosomal microarray analysis by (i) reduced DNA  
56 424 sequencing cost by high throughput sequencing technologies and a high number of  
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3 425 samples can be simultaneously sequenced in a single testing; (ii) enhanced detection  
4 426 of partial or segmental aneuploidies as a result of the increase in chromosomal  
5 427 analysis resolution to a few mega bases; (iii) increased dynamic range enabling  
6 428 enhanced detection of mosaicism in multicellular samples and (iv) automation of the  
7 429 sequencing library preparation and automation of the PGT-A diagnostic procedure.  
8 430 However, the beneficial effect of PGT-A has been nullified by the detrimental effect  
9 431 of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst  
10 432 evaluation tool than the traditional morphology assessment during IVF treatment, it  
11 433 can potentially shorten the time-to-pregnancy in women with infertility.  
12 434

13 435 Currently, all relevant studies on niPGT-A focused on the amplification success and  
14 436 concordance rates between SCM and trophoctoderm biopsy but not on assessing its  
15 437 ability as a screening tool for blastocyst transfer prioritization. This study could  
16 438 provide valuable information on the potential novel use of niPGT-A as an adjunct  
17 439 for morphological assessment of blastocysts.  
18 440  
19 441

#### 20 442 **Trial status**

21 443 The first subject was recruited on 1<sup>st</sup> July 2021 and 400 women were recruited up to  
22 444 the writing of this protocol paper.  
23 445

24 446 **Funding statement:** The study is supported by the Health and Medical Research  
25 447 Fund (Project number 08192196).  
26 448

27 449 **Competing interest:** The authors report no conflicts of interests.  
28 450

#### 29 451 **Contributorship Statement**

30 452 All authors have substantial contribution to the protocol paper:  
31 453

32 454 Cheng Hiu Yee Heidi: Drafting the manuscript and revising it critically for  
33 455 important intellectual content, responsible for seeking ethics approval and  
34 456 registration on ClinicalTrials.gov  
35 457

36 458 Chow, Judy F.C: substantial contribution to designing the work flow and logistics  
37 459 of niPGT-A, responsible for generating niPGT-A report  
38 460

39 461 Lai, Shui Fan: responsible for the recruitment of subjects and seeking ethics  
40 462 approval at the Kwong Wah Hospital  
41 463

42 464 Lam, Kevin KW: responsible for collaborating with the PGT laboratory regarding  
43 465 logistics of collecting SCM and transferral. Responsible for IVF laboratory.  
44 466

45 467 Ng, Ernest HY: Substantial contributions to the conception and design of the whole  
46 468 study, and final approval of the version to be published  
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3 466 Yeung, William Shu Biu: Responsible for the design and worked on logistics of  
4 467 the study, and final approval of the version to be published  
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6 468

7 469 **Full references:**

- 8 1. NICE. Fertility: assessment and treatment for people with fertility  
9 problems. NICE guideline CG11. 2014.
- 10 2. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko  
11 T, Scaravelli G, Smeenk J, Vidakovic S, Goossens V; European IVF-  
12 monitoring Consortium (EIM) for the European Society of Human  
13 Reproduction and Embryology (ESHRE). ART in Europe, 2014: results  
14 generated from European registries by ESHRE: The European IVF-  
15 monitoring Consortium (EIM) for the European Society of Human  
16 Reproduction and Embryology (ESHRE). *Hum Reprod.* 2018 Sep  
17 1;33(9):1586-1601.
- 18 3. Spandorfer SD, Davis OK, Barmat LI, Chung PH, Rosenwaks Z.  
19 Relationship between maternal age and aneuploidy in in vitro fertilization  
20 pregnancy loss. *Fertility and sterility.* 2004;81(5):1265-9.
- 21 4. Hassold T, Hall H, Hunt P. The origin of human aneuploidy: where we have  
22 been, where we are going. *Human molecular genetics.* 2007;16(R2):R203-  
23 R8.
- 24 5. Andersen A-MN, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal  
25 age and fetal loss: population based register linkage study. *BMJ.*  
26 2000;320(7251):1708-12.
- 27 6. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation  
28 genetic screening: a systematic review and meta-analysis of RCTs. *Hum*  
29 *Reprod Update.* 2011 Jul-Aug;17(4):454-66.
- 30 7. Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of  
31 single blastocysts for fresh transfer via standard morphology assessment  
32 alone and with array CGH for good prognosis IVF patients: results from a  
33 randomized pilot study. *Molecular cytogenetics.* 2012;5(1):1-8.
- 34 8. Scott Jr RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al.  
35 Blastocyst biopsy with comprehensive chromosome screening and fresh  
36 embryo transfer significantly increases in vitro fertilization implantation  
37 and delivery rates: a randomized controlled trial. *Fertility and Sterility.*  
38 2013;100(3):697-703.
- 39 9. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, et al. In vitro  
40 fertilization with single euploid blastocyst transfer: a randomized controlled  
41 trial. *Fertility and Sterility.* 2013;100(1):100-7. e1.
- 42 10. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et  
43 al. Development and validation of a next-generation sequencing-based  
44 protocol for 24-chromosome aneuploidy screening of embryos. *Fertility*  
45 *and Sterility.* 2014;101(5):1375-82.
- 46 11. Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T,  
47 Salamonsen LA, Rombauts L. Fresh versus frozen embryo transfer: backing  
48 clinical decisions with scientific and clinical evidence. *Human*  
49 *Reproduction Update.* 2014 Nov-Dec;20(6):808-21.
- 50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

12. Lee E, Illingworth P, Wilton L, Chambers GM. The clinical effectiveness of preimplantation genetic diagnosis for aneuploidy in all 24 chromosomes (PGD-A): systematic review. *Hum Reprod*. 2015 Feb;30(2):473-83.
13. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M, Wells D, Gordon T, Stock-Myer S, Willman S; STAR Study Group. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertility and Sterility*. 2019 Dec;112(6):1071-1079.
14. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertility and Sterility*. 2013;100(3):624-30.
15. Assou S, Aït-Ahmed O, El Messaoudi S, Thierry AR, Hamamah S. Noninvasive pre-implantation genetic diagnosis of X-linked disorders. *Med Hypotheses* 2014;83:506–508.
16. Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertil Steril*. 2017;107(1):220–8.
17. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci USA*. 2016;113(42):11907–12.
18. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril*. 2016;106(6):1312–8.
19. Feichtinger M, Vaccari E, Carli L, Wallner E, Madel U, Figl K, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of concept pilot study. *Reprod BioMed Online*. 2017;34(6):583–9.
20. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod*. 2018;33(4):745–56.
21. Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, Ibarrientos Z, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. *PLoS One*. 2018;13(5): e0197262.
22. Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, et al. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril*. 2018;110(3): 467–75.
23. Capalbo A, Romanelli V, Patassini C, Poli M, Girardi L, Giancani A, et al. Diagnostic efficacy of blastocoel fluid and spent media as sources of DNA for preimplantation genetic testing in standard clinical conditions. *Fertil*

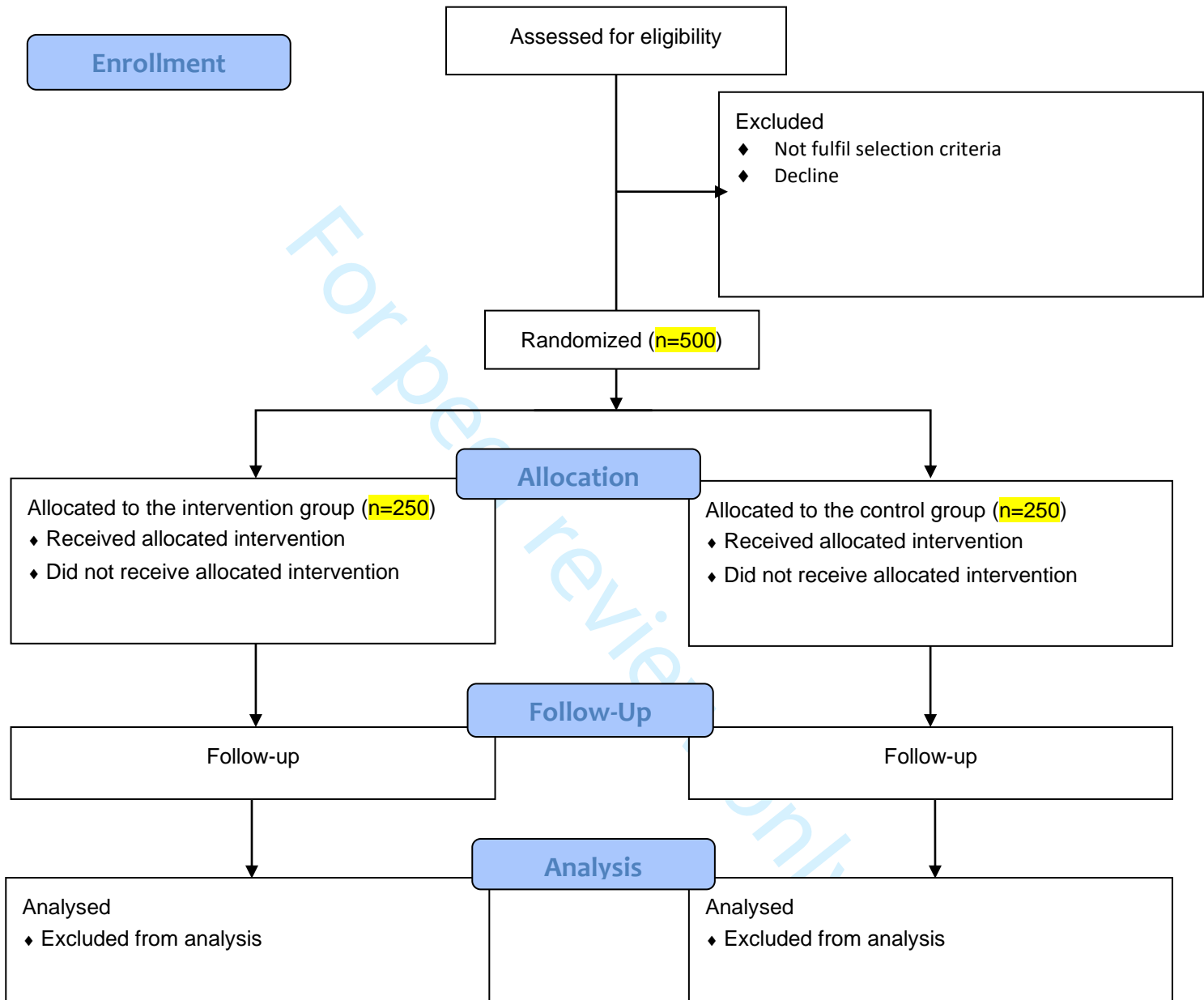
- 1  
2  
3 Steril. 2018;110(5):870–9.  
4  
5 24. Magli M C, Albanese C, Crippa A, et al. Deoxyribonucleic acid detection  
6 in blastocoelic fluid: a new predictor of embryo ploidy and viable  
7 pregnancy[J]. Fertility and sterility, 2019, 111(1): 77-85.  
8  
9 25. Tsai N C, Chang Y C, Su Y R, et al. Validation of Non-Invasive  
10 Preimplantation Genetic Screening Using a Routine IVF Laboratory  
11 Workflow[J]. Biomedicines, 2022, 10(6): 1386.  
12  
13 26. Belandres D, Shamonki M, Arrach N. Current status of spent embryo media  
14 research for preimplantation genetic testing. J Assist Reprod Genet. 2019  
15 May;36(5):819-826.  
16  
17 27. Gardner D, Schoolcraft W, Jansen R, Mortimer D. Towards reproductive  
18 certainty: infertility and genetics beyond. vitro culture of human  
19 blastocysts, Carnforth, Parthenon Press, pp378. 1999;388.

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471 **Word Count: 3387**

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## CONSORT 2010 Flow Diagram





DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY  
THE UNIVERSITY OF HONG KONG

**PATIENT INFORMATION AND CONSENT**

**PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization**

*Version 1.0: Dated 8 March 2020*

You are cordially invited to participate in the above named research study. You need to decide whether you want to participate or not. Please take your time to make up your mind. Carefully read the following and feel free to ask the study doctor any question which you may have.

**Why is this study being done?**

Couples with difficulty conceiving will require test-tube baby or in vitro fertilization (IVF). Despite advances in technology, the pregnancy rate of IVF remained around 35% per transfer. Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. Chromosome aneuploidy is the major reason for failure of pregnancy and miscarriage.

The traditional preimplantation genetic testing for aneuploidy (PGT-A) involves taking a few cells from a blastocyst, which requires skillful laboratory staff and laser equipment. Taking cells from a blastocyst is an invasive procedure and may lead to reduction in implantation potential. The demonstration of release of DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (NIPGT-A). Collection of spent culture medium requires no specialized training and imposes negligible risk to the embryo. Spent culture medium may be more representative of the whole blastocyst.

The results of NIPGT-A will be able to prioritize the sequence of embryo transfer. There is still no good evidence to show the efficacy of NIPGT-A in IVF. The aim of this study is to compare with efficacy of embryo selection for replacement based on conventional method through embryo morphology versus morphology with additional input from result of NIPGT-A.

**Who should be in this study?**

You will be recruited if

1. You are aged less than 43 years at the time of ovarian stimulation, and
2. At least two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval

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You will not be included in this study if

1. Less than two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval;
2. Women undergoing PGT for monogenic diseases or structural rearrangement of chromosomes
3. Use of donor oocytes
4. Hydrosalpinx shown on pelvic scanning and not surgically treated

**What will I be asked to do?**

Recruited patients will undergo IVF as clinically indicated. Ovarian stimulation, ultrasound monitoring, and oocyte retrieval will follow the standard operating procedure of the unit.

Each blastocyst will be frozen individually and its spent culture medium (~8 µl) will be frozen at -80°C separately and individually. The embryologist will prepare a sequence of blastocyst transfer based on the best morphology by Gardner's criteria.

On the day of blastocyst freezing, recruited women will then be randomly assigned into two groups using a randomization program.

1. the intervention group using morphology and NIPGT-A and
2. the control group based on morphology alone.

The women and doctors will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory and the embryologists in the IVF laboratory will be aware of the group assignment.

In the intervention group, comprehensive chromosome screening using NGS will be performed. In the control group, the measurement will be done in the spent culture medium of the blastocyst that is replaced in the first transfer. The NIPGT-A report is used only to prioritize the sequence of embryo transfer. Blastocysts with non-euploid result in the NIPGT-A report will not be discarded.

Blastocysts can be replaced in the subsequent natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. In the control group, blastocysts with the best quality morphology will be replaced first and the sequence of blastocyst transfer is decided prior to randomization. In the intervention group, blastocysts with the best morphology and euploid result will be replaced first as the sequence of blastocyst transfer will be modified after the NIPGT-A reports are available.

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**How long will I be in the study?**

1 IVF cycle

**How many other people will be participating in the study?**

We plan to recruit 500 women in this study.

**Will I be paid?**

No payment will be made to you for this study.

**What adverse (bad) effects can happen to me by participating in the study?**

There should be no major safety concern as all the embryos will be kept after prioritization regardless of the group recruited.

**What benefit can I expect?**

You may have a higher pregnancy rate per transfer, lower miscarriage rate and short time to pregnancy if you are randomized to the intervention group with NIPGT-A.

**Can I refuse to be in the study?**

Your participation in this study is voluntary. You can choose not to take part in the study, or you can quit at any time. You will not lose any benefit to which you are otherwise entitled. If you quit the study, you can receive the standard treatment as other patients in our Department.

**Confidentiality and privacy**

The investigators have always maintained a strict privacy policy. We never sell, trade or otherwise share your details with any sources. All correspondence to the department is held confidentially; furthermore, at no time will your personal and/or identifying information be shared outside of our organization, for any reason.

Subjects have the rights of access to personal data and known study results, if and when needed. Under the laws of the Hong Kong Special Administrative Region and, in particular, the Personal Data (Privacy) Ordinance, Cap 486, you enjoy or may enjoy rights for the protection of the confidentiality of your personal data, such as those regarding the collection, custody, retention, management, control, use (including analysis or comparison), transfer in or out of Hong Kong, non-disclosure, erasure and/or in any way dealing with or disposing of any of your personal data in or for this study. For any query, you should consult the Privacy Commissioner for Privacy Data or his office (2827 2827) as to the proper monitoring or

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4 supervision of your personal data protection so that your full awareness and understanding of  
5 the significance of compliance with the law governing privacy data is assured.  
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8 For questions about the study or reporting of adverse events, please call Dr Heidi Cheng at  
9 telephone no. 22553657. The phone number of the Institutional Review Board of The  
10 University of Hong Kong / Hospital Authority Hong Kong Wester cluster is 2255 4086.  
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14 If you consent to take part in the research, any of your medical records may be inspected by  
15 the research team for purposes of analysing the results. They may also be looked at by  
16 people from regulatory authorities to check that the study is being carried out correctly. Your  
17 name, however, will not be disclosed outside the hospital.  
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THE UNIVERSITY OF HONG KONG

**PATIENT CONSENT FORM**

**PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization**

**Name of Principal Investigator: Dr. Heidi Cheng**

1. I confirm that I have read and understood the patient information sheet for the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
3. I understand that sections of any of my medical notes may be looked at by responsible individuals who are relevant to my taking part in research. I give permission for these individuals to have access to my records.
4. I agree that the spent culture medium may be saved for future research.
5. I agree to take part in the above study.

I understand that I will be given a signed copy of this Patient Information and Consent Form.

\_\_\_\_\_  
Subject's signature

\_\_\_\_\_  
Subject's name

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Date

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Investigator's signature

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Investigator's name

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STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents\*

Section/item	Item No	Description
<b>Administrative information</b>		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym <b>P.1/line 2-3</b>
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry <b>P.2/47-48</b>
	2b	All items from the World Health Organization Trial Registration Data Set <b>P2/47-48</b>
Protocol version	3	Date and version identifier <b>Version 2.0. Date 15 April 2021</b>
Funding	4	Sources and types of financial, material, and other support <b>P.11/line 9-10</b>
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors <b>P.1/line 5-19</b>
	5b	Name and contact information for the trial sponsor <b>Not applicable</b>
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities <b>P11/443-444</b>
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) <b>not applicable</b>
<b>Introduction</b>		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention <b>P3/61 – P5/154</b>
	6b	Explanation for choice of comparators <b>P5/156-159</b>
Objectives	7	Specific objectives or hypotheses <b>P5/162-170</b>

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Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) <b>P5/162</b>
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### **Methods: Participants, interventions, and outcomes**

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained <b>P5/174-177</b>
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Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) <b>P.5/189 – P.6/202</b>
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Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered <b>P6/211-P9/349</b>
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	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) <b>P6/205-207</b>
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	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) <b>Not applicable</b>
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	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial <b>Not applicable</b>
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Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended <b>P.9/352-369</b>
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Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) <b>P6/211-P9/349</b>
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Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations <b>P10/383-393</b>
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Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size <b>P6</b>
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### **Methods: Assignment of interventions (for controlled trials)**

Allocation:

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2	Sequence	16a	Method of generating the allocation sequence (eg, computer-
3	generation		generated random numbers), and list of any factors for stratification.
4			To reduce predictability of a random sequence, details of any planned
5			restriction (eg, blocking) should be provided in a separate document
6			that is unavailable to those who enrol participants or assign
7			interventions <a href="#">P.7/243-247</a>
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10	Allocation	16b	Mechanism of implementing the allocation sequence (eg, central
11	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
12	mechanism		describing any steps to conceal the sequence until interventions are
13			assigned <a href="#">P7/243-249</a>
14			
15	Implementation	16c	Who will generate the allocation sequence, who will enrol participants,
16			and who will assign participants to interventions <a href="#">P7/243-249</a>
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19	Blinding	17a	Who will be blinded after assignment to interventions (eg, trial
20	(masking)		participants, care providers, outcome assessors, data analysts), and
21			how <a href="#">P.7/251-253</a>
22			
23		17b	If blinded, circumstances under which unblinding is permissible, and
24			procedure for revealing a participant's allocated intervention during
25			the trial <a href="#">P.6/204-207</a>
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27			
28	<b>Methods: Data collection, management, and analysis</b>		
29			
30	Data collection	18a	Plans for assessment and collection of outcome, baseline, and other
31	methods		trial data, including any related processes to promote data quality (eg,
32			duplicate measurements, training of assessors) and a description of
33			study instruments (eg, questionnaires, laboratory tests) along with
34			their reliability and validity, if known. Reference to where data
35			collection forms can be found, if not in the protocol <a href="#">P.9/337-349</a>
36			
37			
38		18b	Plans to promote participant retention and complete follow-up,
39			including list of any outcome data to be collected for participants who
40			discontinue or deviate from intervention protocols <a href="#">P9/337-349</a>
41			
42	Data	19	Plans for data entry, coding, security, and storage, including any
43	management		related processes to promote data quality (eg, double data entry;
44			range checks for data values). Reference to where details of data
45			management procedures can be found, if not in the protocol <a href="#">Data will</a>
46			<a href="#">be stored and kept for 10 years, only accessible by investigators as</a>
47			<a href="#">written in the application during ethics approval</a>
48			
49			
50	Statistical	20a	Statistical methods for analysing primary and secondary outcomes.
51	methods		Reference to where other details of the statistical analysis plan can be
52			found, if not in the protocol <a href="#">P.10/396-406</a>
53			
54			
55		20b	Methods for any additional analyses (eg, subgroup and adjusted
56			analyses) <a href="#">P.10/396-406</a>
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20c Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) **P.10/396-406**

### Methods: Monitoring

- Data monitoring 21a Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed **This is not a sponsored trial**
- 21b Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial **No interim analyses will be carried out.**
- Harms 22 Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct **P9/344-349**
- Auditing 23 Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor **Not applicable**

### Ethics and dissemination

- Research ethics approval 24 Plans for seeking research ethics committee/institutional review board (REC/IRB) approval **P.10/408-415**
- Protocol amendments 25 Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) **not applicable**
- Consent or assent 26a Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) **P6/211-213**
- 26b Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable **Not applicable**
- Confidentiality 27 How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial **supplemental material**
- Declaration of interests 28 Financial and other competing interests for principal investigators for the overall trial and each study site **P11/447**

1			
2	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators <b>supplemental material</b>
3			
4			
5			
6	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation <b>Not applicable</b>
7			
8			
9			
10	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions <b>supplemental material</b>
11			
12			
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18		31b	Authorship eligibility guidelines and any intended use of professional writers <b>no intended use of professional writers</b>
19			
20			
21		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code <b>no plans for granting public access</b>
22			
23			
24			
25	<b>Appendices</b>		
26	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates <b>supplemental material</b>
27			
28			
29	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable <b>not applicable</b>
30			
31			
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33			

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\*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.

# BMJ Open

## A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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<b>Primary Subject Heading</b>:	Genetics and genomics
Secondary Subject Heading:	Obstetrics and gynaecology, Reproductive medicine
Keywords:	Subfertility < GYNAECOLOGY, Reproductive medicine < GYNAECOLOGY, GENETICS

SCHOLARONE™  
Manuscripts

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3 1 **Title**

4 2 **A randomised double blind controlled trial of non-invasive preimplantation**  
5 3 **genetic testing for aneuploidy in in vitro fertilisation: a protocol paper**

6 4  
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## 21 **Abstract**

### 22 **Introduction:**

23 The success rate of in vitro fertilisation (IVF) treatment for couples with infertility  
24 remains low due to lack of a reliable tool in selecting euploid embryos for transfer.  
25 This study aims to compare the efficacy in embryo selection based on morphology  
26 alone compared to non-invasive preimplantation genetic testing for aneuploidy  
27 (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation  
28 (IVF).  
29

### 30 **Methods and analysis**

31 This is a randomised double blind controlled trial conducted in two tertiary assisted  
32 reproduction centres. A total of 500 infertile women will be recruited and undergo  
33 IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into  
34 two groups: the intervention group using morphology and niPGT-A and the control  
35 group based on morphology alone. In the control group, blastocysts with the best  
36 quality morphology will be replaced first. In the intervention group, blastocysts with  
37 the best morphology and euploid result of spent culture medium will be replaced  
38 first. The primary outcome is a live birth per the first embryo transfer. The statistical  
39 analysis will be performed with the intention to treat and per protocol.  
40

### 41 **Ethics and dissemination**

42 Ethics approval was sought from the Institutional Review Board of the two  
43 participating units. All participants will provide written informed consent before  
44 joining the study. The results of the study will be submitted to scientific conferences  
45 and peer-reviewed journals.  
46

### 47 **Registration details**

48 The study was registered under ClinicalTrials.gov (trial number NCT04474522).  
49  
50

### 51 **Strengths and limitations of this study**

- 52 • This is a randomised double blind controlled clinical trial.
- 53 • The recruitment takes place in a large representative stakeholder group from  
54 the field of reproductive medicine in Hong Kong.
- 55 • The intervention group of this study includes both morphology and niPGT-A,  
56 thereby investigating the sole effect of addition of niPGT-A on embryo  
57 prioritization alongside with morphology assessment
- 58 • One limitation is that our study is not designed to detect small differences in  
59 the live birth rates.  
60

## 61 Introduction

62 One in seven couples experience difficulty in conceiving. Many of them will require  
63 in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to  
64 stimulate a woman's ovaries to produce a number of oocytes which are collected by  
65 a minor operation and then mixed with sperm to form embryos in the laboratory.  
66 Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage  
67 embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite  
68 advances in ovarian stimulation, culture medium and laboratory conditions, the  
69 pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2).  
70 The corresponding rates in the United States in 2016 were 45% and 36% per transfer  
71 respectively (<https://www.cdc.gov/art/reports/2016/national-summary.html>).

72  
73 The success of IVF depends on selection of the most competent embryos for transfer,  
74 which is still based on morphological criteria by examining the appearance of the  
75 embryos under a microscope. But it is well known that many women fail to achieve  
76 a pregnancy even after transfer of what are perceived to be good quality embryos.  
77 Therefore, some clinics replace multiple embryos in order to maximise pregnancy  
78 rates, a strategy which is associated with a high risk of multiple pregnancy.

79  
80 Chromosome aneuploidy is an error in cell division that results in the "daughter"  
81 cells having the wrong number of chromosomes. In some cases there is a missing  
82 chromosome, while in others an extra one. It is a major reason for failure of  
83 pregnancy, miscarriage and congenital anomalies following both natural conception  
84 and IVF pregnancies and increases exponentially with maternal age (3-5).

85  
86 Our inability to assess embryo quality and select those with the highest potential for  
87 implantation on the basis of morphology has led to the use of preimplantation genetic  
88 testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an  
89 embryo and assessment of the chromosome copy numbers. While PGT-A cannot  
90 create a healthy embryo or improve the quality of an embryo, it provides a method  
91 of selecting embryos with a normal number of chromosomes for transfer. This in  
92 turn has the potential to increase the chance of having a healthy live birth and reduce  
93 the risk of miscarriage or an abnormal fetus caused by an abnormal number of  
94 chromosomes.

95  
96 Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can  
97 only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing  
98 accuracy. A systematic review of nine randomised controlled trials failed to  
99 demonstrate the benefit of the use of PGT-A with FISH(6).

100  
101 Aneuploidy screening of all chromosomes is necessary to determine whether an  
102 embryo is chromosomally normal i.e. comprehensive chromosome screening.  
103 Several small randomized controlled trials showed significantly higher pregnancy or  
104 live birth rate and lower miscarriage rate following the use of using chromosomal  
105 microarray analysis (7-9). The emergence of more advanced genome sequencing  
106 such as next generation sequencing (NGS) provides a reliable, high throughput

1  
2  
3 107 approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week,  
4 108 thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts  
5 109 are frozen post biopsy and the blastocysts with normal genetic makeup are thawed  
6 110 and replaced in a subsequent menstrual cycle. Cryopreservation of blastocysts and  
7 111 replacing the frozen blastocysts after thawing in subsequent cycles become a  
8 112 common practice with vitrification as the cryopreservation method (11).  
9 113

12 114 A systematic review of the clinical utility of PGT-A with comprehensive  
13 115 chromosome screening found that three small randomised controlled trials  
14 116 demonstrated benefit in young and good prognosis patients in terms of clinical  
15 117 pregnancy rates and the use of single embryo transfer (12). However, a recent large  
16 118 randomised controlled trial of 661 women comparing PGT-A using NGS vs  
17 119 morphology showed PGT-A did not improve overall pregnancy outcomes in all  
18 120 women aged 25–40 years with at least two blastocysts that could be biopsied (13).  
19 121 It is possible that there is a detrimental effect of the biopsy of blastocysts on the  
20 122 embryo viability that nullifies the benefit of PGT-A.  
21 123

24 124 The traditional PGT involves biopsy of a few cells from trophectoderm of a  
25 125 blastocyst, which requires skilful laboratory staff and additional instrumentation  
26 126 such as laser equipment. The trophectoderm biopsy is an invasive procedure and  
27 127 may lead to reduction in implantation potential, although the implantation potential  
28 128 is less affected when compared with blastomere biopsy from cleavage stage embryos  
29 129 (14). A non-invasive approach to PGT-A is definitely needed.  
30 130

33 131 The demonstration of release of cell-free DNA from human embryos into the  
34 132 surrounding environment opens up the possibility of non-invasive PGT for  
35 133 aneuploidy (niPGT-A)(15). Collection of spent culture medium (SCM) requires no  
36 134 specialized training and imposes negligible risk to the embryo. SCM may be more  
37 135 representative of the whole blastocyst as embryonic DNA is released from both  
38 136 trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains  
39 137 embryonic DNA from trophectoderm only. Multiple recent studies have  
40 138 demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at  
41 139 the cleavage and blastocyst stages(16-23). It was shown that 24–48 hr of contact  
42 140 with the embryo was sufficient to collect cell-free DNA from SCM (21). The origin  
43 141 of cell-free DNA can be embryonic or parental. It is proposed that the cell-free DNA  
44 142 is derived from cells discarded by the embryos as a corrective mechanism for  
45 143 aneuploidies (16,24). However, the amount of cell-free DNA was not significantly  
46 144 greater in SCM from aneuploid versus euploid embryos, ruling out this possibility  
47 145 (20). Maternal and paternal contamination in SCM can be minimized by performing  
48 146 thorough oocyte stripping and intracytoplasmic sperm injection(25).  
49 147

52 148 In a recent review, the amplification success rate using sequential culture media for  
53 149 SCM ranged between 90% and 100% while the concordance rate in general ploidy  
54 150 i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high as  
55 151 100% with an average of 75% (26). The difference in the general ploidy between  
56 152 SCM and trophectoderm biopsy can be due to mosaicism, which can be revealed in

1  
2  
3 153 trophectoderm biopsy but not in SCM due to the nature of the DNA source and  
4 154 relatively low embryonic DNA fraction.

5 155  
6 156 All relevant studies on niPGT-A focus the amplification success and concordance  
7 157 rates between SCM and trophectoderm biopsy. A large clinical randomised trial is  
8 158 urgently needed to confirm its efficacy in embryo selection during IVF in terms of  
9 159 live birth and miscarriage rates.

10 160

### 11 161 **Objective and hypothesis**

12 162 This randomised double blind controlled trial aims to compare the efficacy in  
13 163 embryo selection based on morphology alone compared to niPGT-A and  
14 164 morphology in infertile women undergoing IVF.

15 165

16 166 Hypothesis to be tested include:

- 17 167 1. The embryo selection based on niPGT-A and morphology results in a higher  
18 168 live birth rate in IVF as compared with that based on morphology alone.  
19 169 2. The embryo selection based on niPGT-A and morphology results in a lower  
20 170 miscarriage rate in IVF as compared with that based on morphology alone.

21 171

### 22 172 **Methods and analysis**

#### 23 173 **Trial design**

24 174 This is a randomised double blind controlled trial. Eligible women seeking fertility  
25 175 treatment in the Assisted Reproduction Units in Department of Obstetrics &  
26 176 Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited  
27 177 for the study and informed written consent will be obtained after counseling.

28 178

29 179 Ethics approval was sought from the Institutional Review Board of the University  
30 180 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
31 181 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
32 182 KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry  
33 183 (Identifier NCT04474522). Written informed consent will be sought before joining  
34 184 the study.

35 185

#### 36 186 **Selection and Withdrawal of subjects:**

37 187 The population for trial will be infertile women undergoing IVF.

38 188

#### 39 189 **Inclusion criteria:**

40 190 Women admitted to the study will fulfil all of the following criteria:

- 41 191 • Age less than 43 years at the time of ovarian stimulation and  
42 192 • Having at least two blastocysts suitable for freezing on day 6 after oocyte  
43 193 retrieval

44 194

#### 45 195 **Exclusion criteria:**

46 196 Women should not be recruited in any of the following conditions:

- 47 197 • Having less than two blastocysts suitable for freezing on day 6 after oocyte  
48 198



- 198 retrieval;
- 199 • Undergoing PGT for monogenic diseases or structural rearrangement of
- 200 chromosomes;
- 201 • Using donor oocytes;
- 202 • Having hydrosalpinx shown on pelvic scanning and not surgically treated

### 203

### 204 ***Withdrawal criteria***

205 Participation in the study is totally voluntary. Recruited women can withdraw from

206 the study at any time without giving any reasons and subsequently they will receive

207 standard medical care.

### 208

### 209 **Treatment of subjects:**

#### 210 Methods

211 Eligible women will be recruited for the study and informed written consent

212 (See online supplemental material) will be obtained after counseling on the

213 day of oocyte retrieval by research nurse or clinicians.

#### 214

#### 215 *IVF protocol*

216 Infertile women will undergo IVF as clinically indicated. They will receive ovarian

217 stimulation as in standard operation procedure. Ovarian stimulation with

218 gonadotropin injections (150-300 IU daily depending on the antral follicle count)

219 will be given. Medroxyprogesterone acetate 10mg daily will be started on the day 2

220 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be started

221 on the day 6 of ovarian stimulation to prevent premature ovulation. Ultrasound

222 monitoring will be performed to monitor the growth of follicles. When three follicles

223 reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel 0.25mg) or

224 GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte retrieval will be

225 scheduled 36 hours after the trigger under transvaginal ultrasound guidance.

226

227 Oocytes will be fertilized conventionally or by intracytoplasmic sperm

228 injection (ICSI) depending on the semen parameters in accordance with the

229 standard operating procedures and normal fertilization will be assessed and

230 confirmed by the presence of two pronuclei. Embryos will be grown

231 individually to the blastocyst stage, up to day 6 after oocyte retrieval, in a

232 monophasic medium. On Day 3, embryos will be rinsed briefly in fresh culture

233 medium. The culture medium will be replenished and culture will be continued

234 at 37°C and 6% CO<sub>2</sub> in reduced oxygen tension (5%). No fresh transfer of

235 blastocysts will be performed in the stimulated cycle.

#### 236

#### 237 *Grading of blastocyst by morphology*

238 Blastocysts are graded according to Gardner's classification (27). Each

239 blastocyst will be cryopreserved on day 6 by vitrification individually and its

240 SCM (~8 µl) will be frozen at -80°C separately and individually. The

241 embryologist will grade the morphology of blastocysts according to Gardner's

242 criteria .

243

244 Then, on day 6 after oocyte retrieval, women will then be randomly assigned  
245 by a PGT laboratory staff into one of the following two groups according to a  
246 computer-generated randomization list with a 1:1 ratio and a block size of 10.  
247 The randomization list will be prepared by a research nurse, who is not  
248 involved in the clinical care of these women.

- 249 1. the intervention group using morphology and niPGT-A and
- 250 2. the control group based on morphology alone.

251

252 The women, clinicians and embryologists in the IVF laboratory will be blinded  
253 to the treatment groups they are assigned. Only the laboratory staff in the PGT  
254 laboratory will be aware of the group assignment.

255

#### 256 *niPGT-A of spent culture medium (SCM)*

257 In the intervention group, comprehensive chromosome screening using NGS  
258 will be performed according to the recommendations of the company in all  
259 SCM samples. In the control group, the measurement will be done  
260 retrospectively after a live birth or when all blastocysts are replaced without a  
261 live birth.

262

263 A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit,  
264 PerkinElmer) will be used to analyse the SCM samples. The protocol has been  
265 previously optimized with non-invasive samples from 15 laboratories around  
266 the world. The kit follows a single tube workflow, two-steps PCR to whole  
267 genome amplification of the DNA in SCM and then attaches indexes and  
268 sequence-specific adapters to template DNA, resulting in sequencing ready  
269 samples.

270

271 After purification, equal molar concentration of indexed DNA from each  
272 sample will be pooled (96 samples) and then sequenced on a MiSeq system  
273 (Illumina) at 1x75 bp read length. On-board secondary analysis will be  
274 performed automatically by the MiSeq Reporter (Illumina) followed by the  
275 PG-Find Software (v 1.0, PerkinElmer). Reads aligning to anomalous,  
276 unstructured and highly repetitive sequences will be filtered from the analysis.  
277 A target bin size of 1,000 kb will be used, giving a minimum resolution of 10  
278 Mb. All genomic positions will be referred to the human genome build NCBI  
279 37.

280

281 According to the default setting of the PG-Find software, classification of  
282 aneuploidy is determined by CNV (copy number variation) value. CNV value  
283  $>2.7$  is considered as gain while CNV value  $<1.3$  is considered as loss. Sample  
284 will be concluded as non-euploid when one or more of the chromosomes show  
285 gain/loss.

286

287 The niPGT-A result of the SCM sample can be euploid, non-euploid and non-  
288 informative. It will be used only to prioritize the sequence of embryo transfer.

1  
2  
3 289 Blastocysts with non-euploid result in the niPGT-A report will not be discarded and  
4 290 will be transferred with lower priority.  
5

6 291

### 7 292 *Blinding*

8 293 The embryologist will grade the morphology of blastocysts according to  
9 294 Gardner's criteria stated above and the grading of blastocysts will be entered  
10 295 into an online database, which will be managed by an IT technician. The  
11 296 laboratory staff in the PGT laboratory will enter the PGT result into an online  
12 297 database when the niPGT-A results are available. The IT technician will merge  
13 298 the data online to compile the sequence of embryo transfer according to a pre-  
14 299 determined algorithm which depends on the day of blastocyst development  
15 300 (day 5 better than day 6), blastocyst morphology and niPGT-A result of the  
16 301 intervention group. The IT technician will issue the sequence of embryo  
17 302 transfer which does not contain information on the grading of the blastocyst  
18 303 and the NIPGT result to the embryologists in the IVF laboratory. Therefore,  
19 304 the subjects recruited, the clinicians and the embryologists will be blinded to  
20 305 the group allocation.  
21  
22  
23  
24  
25

26 306

### 27 307 *A pilot study*

28 308 A pilot study was conducted on 82 SCM from February to September 2020.  
29 309 Media cultured in parallel but without contact with embryos were collected as  
30 310 controls (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and  
31 311 72 SCM resulted in conclusive result (90.0%, 72/80). All controls showed no  
32 312 amplification.  
33

34 313

35 314 In this cohort, 40 SCM with conclusive results were collected from PGT cycles  
36 315 in which trophoctoderm biopsies were also performed. 85.0% (34/40) of  
37 316 samples showed concordance results between trophoctoderm biopsy and  
38 317 SCM.  
39

40 318

### 41 319 *Frozen embryo transfer (FET)*

42 320 Blastocysts will be replaced in the natural or hormonal replacement cycles,  
43 321 depending whether the women have regular menstrual cycles or not. Only one  
44 322 blastocyst will be transferred each time. The embryologists in the IVF laboratory  
45 323 will thaw and transfer the blastocyst according to the sequence of embryo transfer  
46 324 generated and issued by the IT technician. In the control group, blastocysts which  
47 325 develop on day 5 after the oocyte retrieval and have the best grading will be replaced  
48 326 first. In the intervention group, blastocysts which develop on day 5 and have the best  
49 327 grading and euploid result will be replaced first. If no blastocysts have euploid result,  
50 328 those with non-informative followed by non-euploid results will be replaced.  
51

52 329

### 53 330 *Pregnancy*

54 331 A urine pregnancy test will be performed 14 days after the transfer. If the  
55 332 pregnancy test is positive, transvaginal ultrasound will be performed two  
56 333 weeks later to locate the pregnancy and confirm foetal viability and the number  
57 334 of fetuses. Subsequent management will be the same as other women with

1  
2  
3 335 early pregnancy. They will be referred for antenatal care when the ongoing  
4 336 pregnancy is 8-10 weeks.

5 337

6 338 *Follow-up*

7 339 Written consent regarding retrieval of pregnancy and delivery data will be sought  
8 340 from the recruited women at the time of study. The women will be contacted after  
9 341 delivery by phone to retrieve the information of the pregnancy outcomes. The  
10 342 outcome of the pregnancy (delivery, miscarriage), number of babies born, birth  
11 343 weights and obstetrics complications will be recorded.

12 344

13 345 Women in both groups will continue to have blastocyst transfer until all the  
14 346 cryopreserved blastocysts are used up or they become pregnant within 6 months after  
15 347 randomisation. Cumulative live birth rate will be calculated (the number of live birth  
16 348 per couple within the study period). The pregnancy complication and congenital  
17 349 abnormalities of the pregnancies in the two groups will be traced through hospital  
18 350 records or patient contact by mail or phone of mail and compared.

19 351

20 352 **Assessment of outcomes:**

21 353 The primary outcome is live birth beyond 22 weeks of gestation per the first FET.

22 354

23 355 Secondary outcomes include

- 24 356
- 25 357 • Cumulative live birth rate: the number of pregnancies leading to live birth within  
26 358 6 months of randomization.
  - 27 359 • Time to pregnancy
  - 28 360 • Positive urine pregnancy test
  - 29 361 • Clinical pregnancy defined as presence of intrauterine gestational sac on  
30 362 scanning at gestational week 6.
  - 31 363 • Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of  
32 364 gestation
  - 33 365 • Miscarriage defined as a clinically recognized pregnancy loss before the 22  
34 366 weeks of pregnancy and whose denominator is the clinical pregnancy.
  - 35 367 • Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of  
36 368 gestation
  - 37 369 • Ectopic pregnancy
  - 38 370 • Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational  
39 371 diabetes, congenital anomaly, perinatal mortality, Apgar score and birthweight of  
40 372 newborn.

41 372 Consistency of case management:

42 373 The same standardised study protocol will be adopted in the two study centres. The  
43 374 clinicians who manage the women in KWH had all been trained at QMH, and are  
44 375 now adopting the same clinical management protocols in their respective unit.  
45 376 Regular and frequent communication among the two participating centers will also  
46 377 ensure dissemination of updated information on recruitment and safety issues.

47 378

48 379 *Patient and Public involvement*

49 380

1  
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3 380 Patients or the public were not involved in the design, conduct, reporting or  
4 381 dissemination plans of this study.

5 382

## 7 383 **Statistics**

### 8 384 *Sample size calculation*

9 385 From the data in QMH and KWH, the live birth rate following transfer of one  
11 386 blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate  
12 387 following blastocyst morphology and NIPGT-A, the live birth rate will increase from  
13 388 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate  
14 389 observed following conventional PGT-A in the centre. The calculated sample size is  
15 390 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming  
16 391 a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group  
17 392 (Figure 1 – Consort 2010 flow diagram).

18 393

21 394 A pre-specified subgroup analysis will be performed: women aged <35 years vs  
22 395  $\geq 35$  years.

23 396

### 25 397 *Data analysis*

26 398 Demographic features of women in the two groups will be compared. Comparison  
27 399 of quantitative variables will be performed using Student's t or Mann-whitney U-  
28 400 test where appropriate, while categorical variables will be compared using a Chi-  
29 401 square analysis, multivariable logistic regression or the one-way ANOVA test if  
30 402 more than two categories will be compared. All statistical analyses of the data will be  
31 403 performed with the intention to treat and per protocol using the SPSS program  
32 404 V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be considered  
33 405 statistically significant.

34 406

37 407 The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will  
38 408 be calculated.

39 409

## 41 410 **Ethics and dissemination**

42 411 Ethics approval was sought from the Institutional Review Board of the University  
43 412 of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-  
44 413 248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number:  
45 414 KC/KE-20-0098/FR-2). All participants will provide written informed consent  
46 415 before randomization. The research findings from this study will be submitted to  
47 416 scientific conferences and peer-reviewed journals for publication so as to  
48 417 disseminate the results to other researchers and clinicians working in the field.

49 418

## 51 419 **Discussion**

52 420 The success rate of IVF has long been limited due to the inability to assess the  
53 421 implantation potential of each embryo accurately. Assessment by morphology using  
54 422 Gardner's classification could not reflect chromosomal abnormalities in embryos,  
55 423 which is the reason for failure of implantation in most of the cases. NGS offers  
56 424 several advantages over chromosomal microarray analysis by (i) reduced DNA

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3 425 sequencing cost by high throughput sequencing technologies and a high number of  
4 426 samples can be simultaneously sequenced in a single testing; (ii) enhanced detection  
5 427 of partial or segmental aneuploidies as a result of the increase in chromosomal  
6 428 analysis resolution to a few mega bases; (iii) increased dynamic range enabling  
7 429 enhanced detection of mosaicism in multicellular samples and (iv) automation of the  
8 430 sequencing library preparation and automation of the PGT-A diagnostic procedure.  
9 431 However, the beneficial effect of PGT-A has been nullified by the detrimental effect  
10 432 of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst  
11 433 evaluation tool than the traditional morphology assessment during IVF treatment, it  
12 434 can potentially shorten the time-to-pregnancy in women with infertility.  
13 435

14 436 Currently, all relevant studies on niPGT-A focused on the amplification success and  
15 437 concordance rates between SCM and trophectoderm biopsy but not on assessing its  
16 438 ability as a screening tool for blastocyst transfer prioritization. This study could  
17 439 provide valuable information on the potential novel use of niPGT-A as an adjunct  
18 440 for morphological assessment of blastocysts.  
19 441  
20 442

#### 21 443 **Trial status**

22 444 The first subject was recruited on 1<sup>st</sup> July 2021 and 400 women were recruited up to  
23 445 the writing of this protocol paper.

24 446 **Funding statement:** The study is supported by the Health and Medical Research  
25 447 Fund (Project number 08192196).

26 448 **Competing interest:** The authors report no conflicts of interests.

#### 27 449 **Contributorship Statement**

28 450 All authors have substantial contribution to the protocol paper:

29 451 Cheng Hiu Yee Heidi: Drafting the manuscript and revising it critically for  
30 452 important intellectual content, responsible for seeking ethics approval and  
31 453 registration on ClinicalTrials.gov  
32 454

33 455 Chow, Judy F.C: substantial contribution to designing the work flow and logistics  
34 456 of niPGT-A, responsible for generating niPGT-A report  
35 457

36 458 Lai, Shui Fan: responsible for the recruitment of subjects and seeking ethics  
37 459 approval at the Kwong Wah Hospital  
38 460

39 461 Lam, Kevin KW: responsible for collaborating with the PGT laboratory regarding  
40 462 logistics of collecting SCM and transferral. Responsible for IVF laboratory.  
41 463

42 464 Ng, Ernest HY: Substantial contributions to the conception and design of the whole  
43 465 study, and final approval of the version to be published  
44 466

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2  
3 467 Yeung, William Shu Biu: Responsible for the design and worked on logistics of  
4 the study, and final approval of the version to be published  
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7 470 **Full references:**

- 8 1. NICE. Fertility: assessment and treatment for people with fertility  
9 problems. NICE guideline CG11. 2014.
- 10 2. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko  
11 T, Scaravelli G, Smeenk J, Vidakovic S, Goossens V; European IVF-  
12 monitoring Consortium (EIM) for the European Society of Human  
13 Reproduction and Embryology (ESHRE). ART in Europe, 2014: results  
14 generated from European registries by ESHRE: The European IVF-  
15 monitoring Consortium (EIM) for the European Society of Human  
16 Reproduction and Embryology (ESHRE). *Hum Reprod.* 2018 Sep  
17 1;33(9):1586-1601.
- 18 3. Spandorfer SD, Davis OK, Barmat LI, Chung PH, Rosenwaks Z.  
19 Relationship between maternal age and aneuploidy in in vitro fertilization  
20 pregnancy loss. *Fertility and sterility.* 2004;81(5):1265-9.
- 21 4. Hassold T, Hall H, Hunt P. The origin of human aneuploidy: where we have  
22 been, where we are going. *Human molecular genetics.* 2007;16(R2):R203-  
23 R8.
- 24 5. Andersen A-MN, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal  
25 age and fetal loss: population based register linkage study. *BMJ.*  
26 2000;320(7251):1708-12.
- 27 6. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation  
28 genetic screening: a systematic review and meta-analysis of RCTs. *Hum*  
29 *Reprod Update.* 2011 Jul-Aug;17(4):454-66.
- 30 7. Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of  
31 single blastocysts for fresh transfer via standard morphology assessment  
32 alone and with array CGH for good prognosis IVF patients: results from a  
33 randomized pilot study. *Molecular cytogenetics.* 2012;5(1):1-8.
- 34 8. Scott Jr RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al.  
35 Blastocyst biopsy with comprehensive chromosome screening and fresh  
36 embryo transfer significantly increases in vitro fertilization implantation  
37 and delivery rates: a randomized controlled trial. *Fertility and Sterility.*  
38 2013;100(3):697-703.
- 39 9. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, et al. In vitro  
40 fertilization with single euploid blastocyst transfer: a randomized controlled  
41 trial. *Fertility and Sterility.* 2013;100(1):100-7. e1.
- 42 10. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et  
43 al. Development and validation of a next-generation sequencing-based  
44 protocol for 24-chromosome aneuploidy screening of embryos. *Fertility*  
45 *and Sterility.* 2014;101(5):1375-82.
- 46 11. Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T,  
47 Salamonsen LA, Rombauts L. Fresh versus frozen embryo transfer: backing  
48 clinical decisions with scientific and clinical evidence. *Human*  
49 *Reproduction Update.* 2014 Nov-Dec;20(6):808-21.

12. Lee E, Illingworth P, Wilton L, Chambers GM. The clinical effectiveness of preimplantation genetic diagnosis for aneuploidy in all 24 chromosomes (PGD-A): systematic review. *Hum Reprod*. 2015 Feb;30(2):473-83.
13. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M, Wells D, Gordon T, Stock-Myer S, Willman S; STAR Study Group. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertility and Sterility*. 2019 Dec;112(6):1071-1079.
14. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertility and Sterility*. 2013;100(3):624-30.
15. Assou S, Aït-Ahmed O, El Messaoudi S, Thierry AR, Hamamah S. Noninvasive pre-implantation genetic diagnosis of X-linked disorders. *Med Hypotheses* 2014;83:506–508.
16. Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertil Steril*. 2017;107(1):220–8.
17. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci USA*. 2016;113(42):11907–12.
18. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril*. 2016;106(6):1312–8.
19. Feichtinger M, Vaccari E, Carli L, Wallner E, Madel U, Figl K, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of concept pilot study. *Reprod BioMed Online*. 2017;34(6):583–9.
20. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod*. 2018;33(4):745–56.
21. Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, Ibarrientos Z, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. *PLoS One*. 2018;13(5): e0197262.
22. Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, et al. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril*. 2018;110(3): 467–75.
23. Capalbo A, Romanelli V, Patassini C, Poli M, Girardi L, Giancani A, et al. Diagnostic efficacy of blastocoel fluid and spent media as sources of DNA for preimplantation genetic testing in standard clinical conditions. *Fertil*



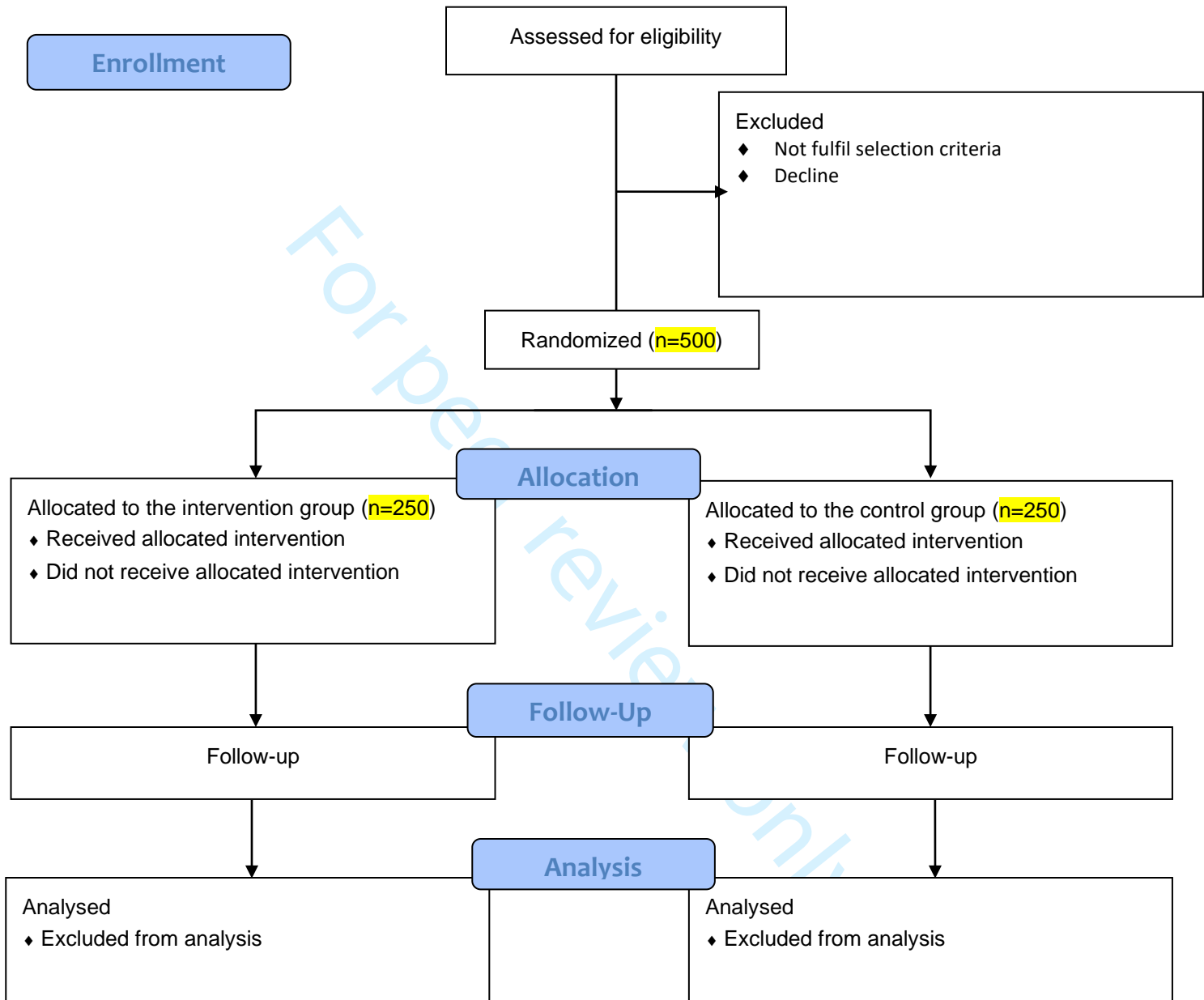
- 1  
2  
3 Steril. 2018;110(5):870–9.  
4  
5 24. Magli M C, Albanese C, Crippa A, et al. Deoxyribonucleic acid detection  
6 in blastocoelic fluid: a new predictor of embryo ploidy and viable  
7 pregnancy[J]. Fertility and sterility, 2019, 111(1): 77-85.  
8  
9 25. Tsai N C, Chang Y C, Su Y R, et al. Validation of Non-Invasive  
10 Preimplantation Genetic Screening Using a Routine IVF Laboratory  
11 Workflow[J]. Biomedicines, 2022, 10(6): 1386.  
12  
13 26. Belandres D, Shamonki M, Arrach N. Current status of spent embryo media  
14 research for preimplantation genetic testing. J Assist Reprod Genet. 2019  
15 May;36(5):819-826.  
16  
17 27. Gardner D, Schoolcraft W, Jansen R, Mortimer D. Towards reproductive  
18 certainty: infertility and genetics beyond. vitro culture of human  
19 blastocysts, Carnforth, Parthenon Press, pp378. 1999;388.

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## CONSORT 2010 Flow Diagram



DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY  
THE UNIVERSITY OF HONG KONG

**PATIENT INFORMATION AND CONSENT**

**PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization**

*Version 1.0: Dated 8 March 2020*

You are cordially invited to participate in the above named research study. You need to decide whether you want to participate or not. Please take your time to make up your mind. Carefully read the following and feel free to ask the study doctor any question which you may have.

**Why is this study being done?**

Couples with difficulty conceiving will require test-tube baby or in vitro fertilization (IVF). Despite advances in technology, the pregnancy rate of IVF remained around 35% per transfer. Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. Chromosome aneuploidy is the major reason for failure of pregnancy and miscarriage.

The traditional preimplantation genetic testing for aneuploidy (PGT-A) involves taking a few cells from a blastocyst, which requires skillful laboratory staff and laser equipment. Taking cells from a blastocyst is an invasive procedure and may lead to reduction in implantation potential. The demonstration of release of DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (NIPGT-A). Collection of spent culture medium requires no specialized training and imposes negligible risk to the embryo. Spent culture medium may be more representative of the whole blastocyst.

The results of NIPGT-A will be able to prioritize the sequence of embryo transfer. There is still no good evidence to show the efficacy of NIPGT-A in IVF. The aim of this study is to compare with efficacy of embryo selection for replacement based on conventional method through embryo morphology versus morphology with additional input from result of NIPGT-A.

**Who should be in this study?**

You will be recruited if

1. You are aged less than 43 years at the time of ovarian stimulation, and
2. At least two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval

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You will not be included in this study if

1. Less than two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval;
2. Women undergoing PGT for monogenic diseases or structural rearrangement of chromosomes
3. Use of donor oocytes
4. Hydrosalpinx shown on pelvic scanning and not surgically treated

**What will I be asked to do?**

Recruited patients will undergo IVF as clinically indicated. Ovarian stimulation, ultrasound monitoring, and oocyte retrieval will follow the standard operating procedure of the unit.

Each blastocyst will be frozen individually and its spent culture medium (~8 µl) will be frozen at -80°C separately and individually. The embryologist will prepare a sequence of blastocyst transfer based on the best morphology by Gardner's criteria.

On the day of blastocyst freezing, recruited women will then be randomly assigned into two groups using a randomization program.

1. the intervention group using morphology and NIPGT-A and
2. the control group based on morphology alone.

The women and doctors will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory and the embryologists in the IVF laboratory will be aware of the group assignment.

In the intervention group, comprehensive chromosome screening using NGS will be performed. In the control group, the measurement will be done in the spent culture medium of the blastocyst that is replaced in the first transfer. The NIPGT-A report is used only to prioritize the sequence of embryo transfer. Blastocysts with non-euploid result in the NIPGT-A report will not be discarded.

Blastocysts can be replaced in the subsequent natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. In the control group, blastocysts with the best quality morphology will be replaced first and the sequence of blastocyst transfer is decided prior to randomization. In the intervention group, blastocysts with the best morphology and euploid result will be replaced first as the sequence of blastocyst transfer will be modified after the NIPGT-A reports are available.

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**How long will I be in the study?**

1 IVF cycle

**How many other people will be participating in the study?**

We plan to recruit 500 women in this study.

**Will I be paid?**

No payment will be made to you for this study.

**What adverse (bad) effects can happen to me by participating in the study?**

There should be no major safety concern as all the embryos will be kept after prioritization regardless of the group recruited.

**What benefit can I expect?**

You may have a higher pregnancy rate per transfer, lower miscarriage rate and short time to pregnancy if you are randomized to the intervention group with NIPGT-A.

**Can I refuse to be in the study?**

Your participation in this study is voluntary. You can choose not to take part in the study, or you can quit at any time. You will not lose any benefit to which you are otherwise entitled. If you quit the study, you can receive the standard treatment as other patients in our Department.

**Confidentiality and privacy**

The investigators have always maintained a strict privacy policy. We never sell, trade or otherwise share your details with any sources. All correspondence to the department is held confidentially; furthermore, at no time will your personal and/or identifying information be shared outside of our organization, for any reason.

Subjects have the rights of access to personal data and known study results, if and when needed. Under the laws of the Hong Kong Special Administrative Region and, in particular, the Personal Data (Privacy) Ordinance, Cap 486, you enjoy or may enjoy rights for the protection of the confidentiality of your personal data, such as those regarding the collection, custody, retention, management, control, use (including analysis or comparison), transfer in or out of Hong Kong, non-disclosure, erasure and/or in any way dealing with or disposing of any of your personal data in or for this study. For any query, you should consult the Privacy Commissioner for Privacy Data or his office (2827 2827) as to the proper monitoring or

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4 supervision of your personal data protection so that your full awareness and understanding of  
5 the significance of compliance with the law governing privacy data is assured.  
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8 For questions about the study or reporting of adverse events, please call Dr Heidi Cheng at  
9 telephone no. 22553657. The phone number of the Institutional Review Board of The  
10 University of Hong Kong / Hospital Authority Hong Kong Wester cluster is 2255 4086.  
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14 If you consent to take part in the research, any of your medical records may be inspected by  
15 the research team for purposes of analysing the results. They may also be looked at by  
16 people from regulatory authorities to check that the study is being carried out correctly. Your  
17 name, however, will not be disclosed outside the hospital.  
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THE UNIVERSITY OF HONG KONG

**PATIENT CONSENT FORM**

**PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization**

**Name of Principal Investigator: Dr. Heidi Cheng**

1. I confirm that I have read and understood the patient information sheet for the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
3. I understand that sections of any of my medical notes may be looked at by responsible individuals who are relevant to my taking part in research. I give permission for these individuals to have access to my records.
4. I agree that the spent culture medium may be saved for future research.
5. I agree to take part in the above study.

I understand that I will be given a signed copy of this Patient Information and Consent Form.

\_\_\_\_\_  
Subject's signature

\_\_\_\_\_  
Subject's name

\_\_\_\_\_  
Date

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Investigator's signature

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Investigator's name

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Witness' name

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Date



STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents\*

Section/item	Item No	Description
<b>Administrative information</b>		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym <b>P.1/line 2-3</b>
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry <b>P.2/47-48</b>
	2b	All items from the World Health Organization Trial Registration Data Set <b>P2/47-48</b>
Protocol version	3	Date and version identifier <b>Version 2.0. Date 15 April 2021</b>
Funding	4	Sources and types of financial, material, and other support <b>P.11/line 9-10</b>
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors <b>P.1/line 5-19</b>
	5b	Name and contact information for the trial sponsor <b>Not applicable</b>
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities <b>P11/443-444</b>
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) <b>not applicable</b>
<b>Introduction</b>		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention <b>P3/61 – P5/154</b>
	6b	Explanation for choice of comparators <b>P5/156-159</b>
Objectives	7	Specific objectives or hypotheses <b>P5/162-170</b>



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Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) <b>P5/162</b>
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### **Methods: Participants, interventions, and outcomes**

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained <b>P5/174-177</b>
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Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) <b>P.5/189 – P.6/202</b>
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Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered <b>P6/211-P9/349</b>
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	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) <b>P6/205-207</b>
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	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) <b>Not applicable</b>
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	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial <b>Not applicable</b>
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Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended <b>P.9/352-369</b>
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Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) <b>P6/211-P9/349</b>
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Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations <b>P10/383-393</b>
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Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size <b>P6</b>
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### **Methods: Assignment of interventions (for controlled trials)**

Allocation:

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2	Sequence	16a	Method of generating the allocation sequence (eg, computer-
3	generation		generated random numbers), and list of any factors for stratification.
4			To reduce predictability of a random sequence, details of any planned
5			restriction (eg, blocking) should be provided in a separate document
6			that is unavailable to those who enrol participants or assign
7			interventions <a href="#">P.7/243-247</a>
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10	Allocation	16b	Mechanism of implementing the allocation sequence (eg, central
11	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
12	mechanism		describing any steps to conceal the sequence until interventions are
13			assigned <a href="#">P7/243-249</a>
14			
15	Implementation	16c	Who will generate the allocation sequence, who will enrol participants,
16			and who will assign participants to interventions <a href="#">P7/243-249</a>
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18			
19	Blinding	17a	Who will be blinded after assignment to interventions (eg, trial
20	(masking)		participants, care providers, outcome assessors, data analysts), and
21			how <a href="#">P.7/251-253</a>
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23		17b	If blinded, circumstances under which unblinding is permissible, and
24			procedure for revealing a participant's allocated intervention during
25			the trial <a href="#">P.6/204-207</a>
26			
27			
28	<b>Methods: Data collection, management, and analysis</b>		
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30	Data collection	18a	Plans for assessment and collection of outcome, baseline, and other
31	methods		trial data, including any related processes to promote data quality (eg,
32			duplicate measurements, training of assessors) and a description of
33			study instruments (eg, questionnaires, laboratory tests) along with
34			their reliability and validity, if known. Reference to where data
35			collection forms can be found, if not in the protocol <a href="#">P.9/337-349</a>
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38		18b	Plans to promote participant retention and complete follow-up,
39			including list of any outcome data to be collected for participants who
40			discontinue or deviate from intervention protocols <a href="#">P9/337-349</a>
41			
42	Data	19	Plans for data entry, coding, security, and storage, including any
43	management		related processes to promote data quality (eg, double data entry;
44			range checks for data values). Reference to where details of data
45			management procedures can be found, if not in the protocol <a href="#">Data will</a>
46			<a href="#">be stored and kept for 10 years, only accessible by investigators as</a>
47			<a href="#">written in the application during ethics approval</a>
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50	Statistical	20a	Statistical methods for analysing primary and secondary outcomes.
51	methods		Reference to where other details of the statistical analysis plan can be
52			found, if not in the protocol <a href="#">P.10/396-406</a>
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55		20b	Methods for any additional analyses (eg, subgroup and adjusted
56			analyses) <a href="#">P.10/396-406</a>
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20c Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) **P.10/396-406**

### Methods: Monitoring

- Data monitoring 21a Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed **This is not a sponsored trial**
- 21b Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial **No interim analyses will be carried out.**
- Harms 22 Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct **P9/344-349**
- Auditing 23 Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor **Not applicable**

### Ethics and dissemination

- Research ethics approval 24 Plans for seeking research ethics committee/institutional review board (REC/IRB) approval **P.10/408-415**
- Protocol amendments 25 Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) **not applicable**
- Consent or assent 26a Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) **P6/211-213**
- 26b Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable **Not applicable**
- Confidentiality 27 How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial **supplemental material**
- Declaration of interests 28 Financial and other competing interests for principal investigators for the overall trial and each study site **P11/447**

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2	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators <b>supplemental material</b>
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6	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation <b>Not applicable</b>
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10	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions <b>supplemental material</b>
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18		31b	Authorship eligibility guidelines and any intended use of professional writers <b>no intended use of professional writers</b>
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21		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code <b>no plans for granting public access</b>
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25	<b>Appendices</b>		
26	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates <b>supplemental material</b>
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29	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable <b>not applicable</b>
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\*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.