
Book Section:
Baker, D. and Barbaric, I. (2022) Characterizing the genetic stability of human naïve and primed pluripotent stem cells. In: Rugg-Gunn, P., (ed.) Human Naïve Pluripotent Stem Cells. Methods in Molecular Biology (2416). Humana Press (part of Springer Nature) , pp. 267-284. ISBN 9781071619070

https://doi.org/10.1007/978-1-0716-1908-7_17

Characterising the genetic stability of naïve and primed human pluripotent stem cells

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ABSTRACT

The presence of genetic changes in human pluripotent stem cells (hPSCs) can affect their behaviour and impact on the utility of hPSC-based applications in research and clinic. The spectrum of spontaneously arising genetic abnormalities in hPSCs is wide and ranges from numerical and structural chromosomal anomalies down to point mutations. The detection of genetic changes in hPSCs is confounded by the fact that no single method detects all types of abnormalities with the same accuracy and sensitivity, therefore necessitating the use of a combination of different methods. Here, we provide detailed protocols for two methods commonly utilised for the detection of genetic changes in naïve and primed hPSCs: karyotyping by G-banding and Fluorescent In Situ Hybridisation (FISH).

Keywords: Human Pluripotent Stem Cells (hPSCs), Genetic Changes, Aneuploidy, Polyploidy, G-banding, FISH

Running title: Genetic stability of human pluripotent stem cells

1 INTRODUCTION

The euploid, non-transformed and non-immortalised character of human pluripotent stem cells (hPSCs) [1, 2] has been touted as a key advantage of using these cells in basic research, and it is considered an essential pre-requisite for their use in regenerative medicine. Accordingly, the occurrence of genetic changes that could potentially compromise this defining character of hPSCs presents a major obstacle for their application in research and clinic [3]. Although hPSCs can be maintained in a euploid state for extensive periods of time, their genomes can also succumb to genetic changes upon culture [4, 5, 6, 7]. This is true for both naïve and primed hPSCs, although patterns of abnormalities detected seem to differ between these two states of pluripotency. In the primed state, genetic changes are known to be non-random, with recurrently acquired aberrations entailing gains of whole or parts of chromosomes 1, 12, 17, 20 and X [5,8], or mutations in *TP53* [6] and several other cancer-related genes [7]. In the naïve state, the extent of potential commonalities of genetic aberrations is still unclear, but polyploidy seems relatively common, at least under some of the culture conditions for deriving and maintaining naïve hPSCs [9, 10]. Moreover, aneuploidy and point mutations were also detected in naïve hPSCs [7,9,10], demonstrating that genetic aberrations in hPSCs, be it naïve or primed, encompass a whole spectrum of abnormalities, from gross karyotypic abnormalities down to point mutations.

Genetic changes can alter hPSC phenotype and behaviour [11], thus contributing to the variability in protocols and impacting on the data interpretation, robustness and reproducibility. Hence, hPSC cultures must be regularly examined for the presence of genetic changes. Critical considerations in monitoring of hPSC cultures for the presence of variant cells are the frequency of monitoring and the choice of the genotyping assay. Although there are no prescribed rules for the frequency of hPSC testing, the observation that variant hPSCs

can rapidly overtake the cultures [4,12,13] or, in some cases, even eliminate the wild-type counterparts [14] highlights the need for surveillance of expanding hPSC cultures at least every 5-10 passages. Such regular monitoring should also be supplemented with genotyping of lines after they had been exposed to stressful conditions, for example after thawing, single cell cloning or genetic modifications. A confounding factor in choosing the genotyping assay is the fact that none of the existing detection methods are suitable for identifying all types of genetic changes with an equal resolution, sensitivity and speed [8]. For example, whilst karyotyping allows assessment of the entire chromosome complement in a single assay, it has a limited resolution, meaning that it typically detects aberrations only if they are larger than ~5-10Mb [15]. On the other hand, FISH can detect focal amplifications and deletions, but it does so for predetermined loci, rather than providing a whole genome overview. Consequently, in assessing the presence of genetic changes in hPSC cultures, combinations of methods should be utilised, making the routine assessment of hPSC cultures more challenging in terms of the feasibility, throughput, time and costs. Here, we provide detailed protocols for two commonly utilised methods for the detection of genetic abnormalities in hPSC: karyotyping by G-banding and Fluorescent In Situ Hybridisation (FISH). We highlight common technical difficulties in using these methods and provide suggestions for troubleshooting.

2 MATERIALS

2.1. Harvesting hPSC cultures for karyotyping and FISH

1. Sub-confluent hPSC culture (at around 50-70% confluency), containing actively dividing cells.

2. Colcemid solution: Dilute concentrated colcemid to 2 µg/mL with sterile phosphate buffered saline from a pre-prepared solution (e.g. KaryoMAX Colcemid, Thermo Fisher Scientific).
3. Trypsin solution (2.5 %). Trypsin may be stored in a fridge for approximately 1 week. Batches of trypsin solution may be stored at -20 ^{o}C and should be thoroughly defrosted before use.
4. Carnoy's fixative: add 1-part acetic acid to 3-parts of absolute methanol. Fixative should be made fresh before each use.
5. 37.5 mM potassium chloride (KCl) solution: Dissolve 2.79g KCl in distilled water and make up to 1 L.

2.2. Slide preparation

1. Glass microscope slides.
2. Carnoy's fixative: add 1-part acetic acid and 3-parts of absolute methanol.
3. Blotting paper.

2.3. Aging of slides and G-banding

1. Glass microscope slides.
2. Gurr's buffer, pH 6.8: A convenient way is to use commercially available buffer tablets (e.g. from (Gibco)), which are then simply dissolved in water. Dissolve one tablet in 1 L of distilled water.
3. Leishman's solution: Add 3.5 g of Leishman's powder to 2 L of methanol in a flask. Plug the neck of the flask with cotton wool and place on a magnetic stirrer for 4-5 hours. Filter into bottles through Whatman filter paper. Make up in a 1 to 4 ratio with Gurr's buffer before staining slides.

4. Sorenson's buffer, pH 7.0: dissolve 18.16 g of KH_2PO_4 and 18.94g of Na_2HPO_4 in ultra-pure water and make up to 2 L. Mix using a magnetic stirrer until completely dissolved.
5. Trypsin solution: dissolve 2.4 g trypsin in 2 L Sorenson's buffer.
6. Carnoy's fixative: add 1-part acetic acid and 3-parts of absolute methanol.
7. Ethanol.
8. Fibre-free blotting paper.
9. Glass coplin jars.

2.4. FISH

1. Microscope slides with testing material present.
2. FISH probes: probes are available from a number of commercial suppliers. They are typically supplied in batches of 10 to 20 tests and include control probes. FISH probes should be stored at -20 ^{o}C.
3. Coverslips 9 mm, 13 mm, 16 mm, 22 x 22 mm 22 x 32 mm or 22 x 50 mm.
4. Rubber solution (e.g. Weldtite).
5. Fibre-free blotting paper.
6. 20 x Saline sodium citrate (SSC): 3M sodium chloride and 0.3M sodium citrate. We use a commercially available solution (e.g. from Vysis (Abbott Molecular Inc.)).
7. 0.4 x SSCT post hybridisation wash solution: combine 970 mL of distilled water, 20 mL of 20xSSC and 3 mL of NP40 or Tween 20. Mix together and make volume up to 1 L.
8. 2 x SSCT post hybridisation wash solution: combine 894 mL of distilled water, 100 mL of 20xSSC and 1 mL of NP40 or Tween 20. Mix together and make volume up to 1 L.

9. Nail varnish.
10. Ethanol series of 70 %, 95 % and 100 % ethanol.
11. DAPI counterstain: add 100 µl DAPI/Antifade (Vector laboratories) to 900 µl Antifade Mounting Medium (Vector laboratories). Store at 4 ^{o}C. Keep light exposure to a minimum.
12. Plastic coplin jars.
13. Glass coplin jars.
14. Hotplate.
15. Fluorescence microscope with dual or triple band pass filters and a 100X objective.

3 METHODS

3.1. Harvesting cell cultures for karyotyping and FISH

Harvesting metaphase cells for karyotype or FISH is a three-stage process entailing mitotic arrest, hypotonic treatment and cell fixation. This protocol performs optimally for sub-confluent cultures that are approximately 3-4 days post passage and have recently been fed with fresh medium. This maximises the chance of having cells in an exponential phase of growth, therefore promoting higher numbers of cells in metaphase.

Mitotic arrest: The purpose of this stage is to maximise the number of metaphase cells for analysis. Colcemid is the most commonly used mitotic arrestant. Colcemid stops cell division at metaphase by binding to tubulin proteins, obstructing formation of spindle fibres and arresting cell division. Colchicine may be used instead of colcemid, but is slower acting.
Hypotonic treatment: The osmotic pressure introduced by the hypotonic solution, KCl, causes cells to swell allowing chromosomes to spread out. If the hypotonic stage is skipped,

the chromosomes will not be spread out sufficiently for analysis. In addition, the interphase cells will appear substantially smaller**.**

Fixation: Using a 3:1 methanol:acetic acid solution, the cells are fixed in a swollen state over three fixations. Cell debris is gradually removed through repeated fixations. Without sufficient fixation there would be too much cell debris remaining, appearing as a shadow surrounding the chromosomes and preventing optimum G-banding and FISH analysis.

1. Pre-warm the KCl and trypsin solutions in a 37 ^{o}C incubator or a water bath.
2. Add 2µg/mL colcemid solution to a culture flask for a final concentration of 0.08 µg/mL colcemid (e.g. add 0.4 mL of 2 µg/mL colcemid solution to 10 mL of culture medium or 0.2 mL to 5 mL culture medium). Incubate for 4 hours at 37 ^{o}C (*see* Note 1).
3. Transfer the medium into a 10 mL test tube and centrifuge for 8 min at 100 x g. Remove the supernatant from the pellet (*see* Note 2).
4. In the meantime, add 1 mL of pre-warmed trypsin to the flask of cells and incubate for about 4 or 5 min at 37 ^{o}C, or until the cells have detached from the surface of the culture vessel (check this using an inverted microscope). Detaching cells from the flask surface may require some gentle encouragement by tapping the underside of the flask. Note, however, that cells in metaphase would be expected to have detached comparatively easily.
5. Transfer all of the detached cells from the flask into the test tube from step 3 and transfer half of this suspension to a second test tube (splitting the sample into two tubes produces a cleaner preparation).
6. Re-suspend the cells in 8 mL of pre-warmed (to 37 ^{o}C) 37.5 mM KCl solution.
7. Centrifuge cells at 100 x g for 8 min.
8. Remove supernatant using a vacuum pump or pipette in Class II safety cabinet, leaving the cell pellet in approximately 0.5 mL liquid in the tube, and carefully re-suspend cells.

9. Add 4 mL of Carnoy's fixative, carefully agitating the tube as you do so. The first 2 mL of the fixative should be added drop by drop with constant gentle agitation of the suspension to keep it moving, by constant finger tapping the end of the tube. The remaining 2 mL of fixative may be added at a faster rate, with 0.5 mL additions of fixative, again with constant agitation (*see* Note 3).
10. Centrifuge cells at 100 x g for 8 min.
11. Remove most of the supernatant using a vacuum pump, leaving the cell pellet in about 0.5 mL liquid. Carefully re-suspend the cells by gently flicking the pellet.
12. Repeat fixation steps 9-11 twice more. Note that the agitation and slow addition of fixative is not necessary for the second and third fixations.
13. Either make slides straight away (3.2) or store samples at -20 until ready. Better quality preparations and G-banding are produced if samples are left at -20 ^{o}C overnight.

3.2. Slide preparation

The aim of slide making is to produce a sufficient number of metaphases for analysis. The chromosomes of each cell need to be sufficiently spread and non-overlapping so that chromosomes may be distinguished from one another. *See* Note 4 for more detail regarding preparation quality.

1. Place the slides in ice-cold water prior to using them for preparing chromosome spreads.
2. Centrifuge the fixed cell suspension at 100 x g for 8 min.
3. Remove supernatant, leaving the cell pellet in about 0.5 mL liquid, and carefully re-suspend the cells. If the suspension is too dense it will appear milk white. In this case, add extra fixative to dilute the cell suspension until only slightly cloudy.
4. Using a 1 mL pipette, apply one drop of the cell suspension from a height of 1 - 2 inches onto clean, wet, ice-cold slides (2 slides per culture). Blot off excess liquid by touching a

blot paper with one end of the slide, then use a 1 mL pipette to add a drop of Carnoy's fixative to each slide in order to enhance the chromosome spreading (*see* Note 5).

5. Leave the slide for 5 min to dry and proceed to staining (3.3 or 3.4).

3.3. G-banding

To visualise chromosome preparations for detailed analysis, G-banding is the most commonly used staining technique. This technique has been named after the Giemsa dye, but chromosome banding can also be produced with other dyes, such as Leishmann's. In G-bands, the dark regions tend to be heterochromatic, late-replicating and AT-rich. The bright regions tend to be euchromatic, early-replicating and GC-rich. The protocol described here uses Leishman's solution for G-banding. If using Giemsa, the slide making, slide aging and trypsin time will be the same, however, the stain preparation and stain time may vary.

It is important to note that the times stated below for slide aging, trypsin exposure and staining are given as a starting point. Each laboratory should trial variations with these times to find the optimum for their preparations.

3.3.1. Aging of slides and G-banding

1. Fill separate glass coplin jars with the following solutions: Leishman's solution, Gurr's buffer, Carnoy's fixative and ethanol (*see* Note 6).
2. Submerge the slide in Leishman's solution for 1 min.
3. Wash the stain off by immersing the slide in a coplin jar containing Gurr's buffer.
4. De-stain the slide in Carnoy's fixative in a glass coplin jar for 2 min, then rinse in ethanol and leave the slide to dry at room temperature.
5. Age the slide by leaving it overnight in a 60 ˚C oven or on a 97 ˚C hotplate. Aging the slide prepares the chromosomes for banding. By aging the slide, chromosomes appear

crisp and clear. Under-aged slides can appear blurry or 'fuzzy' and do not band well (*see* **Table 1**). The older the slide is, the shorter the incubation period required. Urgent slides can be incubated for shorter times after a stain and de-stain treatment (steps 2 - 4). Once aged, make sure the slide has cooled to room temperature before proceeding.

6. Fill up a glass coplin jar with trypsin solution and place in an incubator or a water bath to warm up to 37 ˚C.
7. Fill three additional coplin jars with the following solutions: Sorenson's buffer, Leishman's solution and Gurr's buffer.
8. Submerge the slide in the trypsin solution and leave for around 25 - 30 seconds. You may need to optimise the exact duration of trypsin exposure suitable for your preparations. Refer to **Table 1** for troubleshooting tips related to trypsin exposure.
9. Rinse off the trypsin by submerging the slide in a coplin jar with Sorenson's buffer. Shake the excess liquid off the slide.
10. Submerge the slide in the Leishman's solution for around 1.5 to 2 min. You may need to trial some slides to find the optimum time for your preparations.
11. Rinse off the stain with Gurr's buffer by submerging the slide in a coplin jar and shake the excess liquid off the slide.
12. Gently place the slide onto a clean, dry paper towel and blot with fibre-free blotting paper.
13. Check the slide for quality and refer to **Table 1** and Note 7 for a troubleshooting guide and tips for improvement.

3.3.2. G-banding analysis

1. Analysing G-banding metaphases requires a high level of training and experience. To analyse G-banded chromosomes, images of metaphases are taken. The images of

individual chromosomes within a metaphase are cut out and arranged in homologous pairs, following a standardized format [15], to create a karyogram.

2. A karyogram is examined for the ploidy levels (i.e. the number of haploid sets of chromosomes; two haploid sets, 46 chromosomes, is normal diploid. Three haploid sets, 69 chromosomes is triploid) and a presence of numerical or structural aberrations. In a clinical and research setting cytogenetic analysis will typically involve examination of a minimum of 20 metaphases per sample where mosaic cell populations are expected. This is expected to detect cell populations that are present in 10-20% of cells [8]. Abnormalities are recorded if the same numerical or structural aberration is seen in two or more cells, or if the same chromosome loss is seen in three or more cells with the sample. Karyotypes are written according to established International System for Human Cytogenetic Nomenclature (ISCN) [15]. For example, 46,XY[20] denotes a male population of cells with no abnormalities found in 20 metaphases analysed. Karyotype 47,XX,+12[20] denotes a female population of cells with a gain of chromosome 12 in all 20 metaphases analysed. Karyotype 47,XX,+12[10]/46,XX[10] denotes a mosaic population of cells, with ten of the cells containing a gain of chromosome 12, whilst the remaining ten cells have a normal female karyotype.
3. When analysing hPSC karyotypes, look out for recurrent genetic changes in primed hPSCs, such as trisomy 12 (Fig.2A), or for presence of polyploidy in naïve hPSCs (Fig.2B).

3.4. Fluorescence in situ hybridisation (FISH)

Fluorescence in situ hybridisation (FISH) offers a rapid, sensitive, and specific detection and characterization of chromosome abnormalities. In this method, fluorochrome labelled DNA probes are hybridised to complementary target DNA sequences. FISH is used to test

metaphase chromosomes and/or interphase cells from cultured samples. The protocol described here uses commercial FISH probe kits, which produce two signals in normal cells – one for the location of interest (test signal) and a second one specific for a different region on the same chromosome, such as the centromere (control signal). If a test signal is absent, the presence of the control signal confirms that the FISH protocol has worked and that the absence of the test signal is due to a loss of the test region, e.g. due to a deletion.

The FISH procedures are divided into following stages: i) probe application; ii) denaturation of probe and target DNA; iii) hybridisation of target with probe; iv) post-hybridisation wash, and v) detection. It is important to note that fluorochrome labelled probes are light sensitive. Therefore, all steps in the following protocols that involve fluorochrome labelled probes should be performed with minimum light exposure.

3.4.1. FISH slide assessment

1. Observe the slides from section 3.2 under the light microscope with a 100X magnification.
2. Check whether the distribution of cells on the slide is appropriate (**Table 2**). Ideally, at least 4 metaphases must be seen in one plane of view. If there are less than 4 metaphases then more material may be added by applying another drop of fixed cell preparation (section 3.2.4).
3. Probes are expensive so it is worthwhile limiting the area that the probe is applied to and selecting a suitably sized coverslip. *See* Notes 8 and 9 and **Table 2** for further details on slide assessment and troubleshooting tips.

3.4.2. Probe Application

1. If using a commercial FISH probe at 100 % concentration, hand warm the probe to room temperature, vortex and pulse in a centrifuge. **Do not warm in a 37 ^{o}C water bath.**
2. For diluted commercial probes, warm to 37 ^{0}C in a water bath for a few seconds then vortex and pulse in centrifuge before pipetting immediately.
3. Immediately apply the probe to an appropriately sized coverslip (25 µl on 25 x 50 mm, 18 µl on a 32x22 mm, 10 µl on a 22x22 mm, 4.4 µl on a 16 mm diameter, 3.3 µl on a 13 mm diameter, or 2.2 µl on a 9 mm diameter). *See* Note 10.
4. Gently overlay the slide with the coverslip and wait for the probe to spread out to the edges of the coverslip (do not press down as this will force the probe out of the edges of the coverslip). If detritus prevents the probe spreading, leaving air pockets, remove the coverslip and re-apply a new coverslip and probe.
5. Seal with rubber solution and air-dry for a few minutes.

3.4.3. Denaturation and hybridisation

1. The probe and target DNA can be denatured together on a 73 °C hotplate for 5 minutes (*see* Note 11).
2. Alternatively, the probe may be denatured in an Eppendorf tube in a 73 °C water bath and the slide on the hotplate before probe application for 5 minutes.
3. Following denaturation, the slide should be immediately transferred to a 37 °C incubator. It is important to place the slide on wet fibre-free blotting paper to ensure the probe does not dry out during hybridisation.
4. Slides may be left in the incubator for hybridisation overnight, and can be left over a weekend. A minimum of 6 hours hybridisation time is recommended.

3.4.4. Post Hybridisation Wash

1. Transfer approximately 50 mL of 0.4 x SSCT post-hybridisation wash solution into a labelled plastic coplin jar (one jar for every four slides to be processed) and place in a 73 °C water bath. Check that the water bath water level is two-thirds up the outside of the coplin jars to ensure that all of the hybridised area on the slides is exposed to the correct temperature.
2. Transfer approximately 50 mL of 2 x SSCT post-hybridisation wash solution into an additional, labelled plastic coplin jar (one for every three slides to be processed) and leave on a bench (usually overnight to warm up to room temperature).
3. Remove the rubber solution and coverslip(s) using forceps. Coverslips should be easy to remove if sufficient probe has been applied and they have not dried out. If particular coverslips are difficult to remove, these slides will need to be reviewed immediately upon completion of processing, as cellular material may have been lost and/or poor hybridisation may have been achieved in certain areas.
4. In a fume cupboard, transfer the slide (a maximum of 4 slides per coplin jar) to the 73 °C 0.4 x SSCT post-hybridisation wash solution and return the coplin jar quickly to the 73 °C water bath to leave to wash for 2 mins. It is important that this time does not exceed 2 mins as it will diminish signal strength on analysis.
5. Remove the slide from the water bath and transfer the slide to the 2 x SSCT post-hybridisation wash solution and wash for 30 seconds to 2 min at room temperature.
6. Dehydrate the slide through an ethanol series of 70%, 95% and 100% ethanol for 2 min each.
7. Air-dry the slide in darkness.
8. In a fume cupboard, apply 20 µl DAPI-containing mounting medium to a 22 x 50 mm coverslip and gently overlay the slide. Wait for the mounting medium to spread out to

the edges of the coverslip (do not press down as this will force the medium out of the edges of the coverslip). Seal the edges with nail varnish (to prevent the sample from drying) and air-dry the varnish.

9. Once the varnish is dry, the slides are ready for analysis. FISH slides should be stored in a fridge, to prevent the DAPI drying out.

3.4.5. FISH analysis

1. Use a 10X objective to locate the cells and then increase magnification to 100X to visualise the FISH signals and perform the analysis.
2. Observe at least 30 metaphases or 100 interphase cells to detect the presence and proportion of abnormal cells within the preparation.
3. FISH analysis involves scoring cells for the number of fluorescent signals. Cells (metaphase or interphase) should have clear separation from neighbouring cells to avoid any confusion over which cell each FISH signals belong to.
4. FISH signals should be distinct from neighbouring signals and be clearly distinguishable from any background fluorescence.
5. Analysis of interphase cells is recommended for scoring large numbers of cells. If identifying the chromosomal location of the FISH probe signal is required, then analysis of metaphase chromosomes is necessary.
6. Examples of good and poor FISH preparations are shown in Fig.3. *See* Note 12 for troubleshooting guide if FISH signal is too faint.

4 NOTES

1. Colcemid prevents the formation of the mitotic spindle and, hence, colcemid treatment leads to arresting cells in metaphase. The longer the exposure time to colcemid, the greater

the number of arrested cells. Nonetheless, colcemid also causes chromosome contraction, which can make analysis of individual chromosomes difficult. Therefore, it is important to optimise the colcemid treatment time to achieve a suitable mitotic index and adequate chromosome length.

2. During mitosis, hPSCs round up and are loosely attached to the culture flask surface. Harvesting the medium prior to trypsin treatment of the adherent cells ensures that loosely attached or detached mitotic cells in the culture medium are retained in the preparation.

3. It is important to perform this first fixation step slowly. If fixation is performed too fast, cells will lyse and chromosomes will be lost. There would be few metaphases present for analysis.

4. Guide for quality assessment of slides:

Mitotic Index (MI) – a measure of the number of metaphases present on a slide

- Good: Abundance of metaphases observed (e.g. 5 metaphases observed in a single field of view at 100X magnification using a light microscope).
- OK: Few metaphases observed (e.g. 1 - 2 metaphases observed in a single field of view at 100X magnification using a light microscope).
- Poor: No metaphases observed.

Chromosome Spreading

- Good: Chromosomes well spread out, with little overlap of individual chromosomes and well-defined chromosome boundaries. No cytoplasm observed. An example is shown in Fig.1A.
- OK: Chromosomes spread out but considerable overlapping. No cytoplasm observed. An example is shown in Fig.1B.

- Poor: Cytoplasmic encapsulated metaphase enveloped by cytoplasm; appears like a round ball containing all chromosomes. Chromosomes are not well defined, and hence analysis is impossible. Examples are shown in Fig.1C and Fig.1D.

5. The spreading of chromosomes is influenced by both the height that the suspension is dropped from and the time that it takes for the fluid to evaporate on the slide. If the fluid evaporates too fast, then chromosomes will not spread out sufficiently. If the fluid evaporates too slowly, then chromosomes will disperse to such a degree that chromosomes from different cells will be intermixed, thereby hindering analysis. The height from which the cells are dropped is also critical. We find that the height should be no more than 2 inches; higher than this and chromosomes may spread too far and be lost from cells. However, if the height is too low the cell membrane may not burst, which would prevent spreading of the chromosomes.

6. Instead of using coplin jars containing the solutions, staining can be done using a slide rack over a sink. It is important to ensure that the slide is covered in the required solutions.

7. Troubleshooting metaphase preparations:

No/low numbers of metaphases on the slide:

- Was colcemid solution added?
- Was the first fixation step performed too fast, lysing cells?
- Was an incorrect KCl concentration used, leading to burst cells and chromosomes lost?
- Were there any actively dividing cells in the culture? Confluent cultures have less actively dividing cells, hence it is best to perform harvesting 1 - 2 days post subculture/passage, when cells are actively proliferating.
- If there are also few interphases present then the suspension may be too dilute. In this case, centrifuge the sample and make slides from a more concentrated suspension.

Chromosomes are poorly spread:

- Was the KCl solution stage missed?
- Try making slides on wet paper, to slow the drying time, allowing for additional spreading of chromosomes.
- Drop the suspension on to the slide from a greater height (*see* Note 4).

Too much cytoplasm surrounding chromosomes.

- Perform fixation again, until the cytoplasm is reduced sufficiently.

8. If slides are over 7 days old, try to obtain fresh slides if there is remaining sample available.

9. There are occasions when the fixed material appears unclean when viewed under the microscope. If this occurs then the material will need to be re-fixed in order to clean up the preparation and new slides should be prepared. Otherwise, the hybridisation may be poor (faint signals) or too much background hybridisation reducing quality (becomes difficult to separate true signal from background).

10. Do not leave probes on the bench without a light-proof cover. Use lidded light-proof coplin jars during processing and air-dry slides under a light-proof cover.

11. FISH probes contain formamide. This lowers the melting temperature, i.e. the temperature at which 50% of duplex molecules become disassociated.

12. Troubleshooting FISH

If FISH signal is too faint:

- Fluorescent signal strength does vary between individual FISH tests and poorer signal strength does necessarily signify an assay problem. Typically, the best first step is to repeat the FISH without altering any processes, but be sure to use fresh wash solutions for every FISH run.

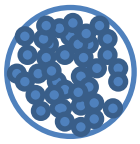
- Check the FISH probe. When using the last aliquots of a FISH probe it has been noted that quality can suffer. Using a new batch of probe often helps.
- Check whether other FISH probes give a similar result.
- Check whether the microscope filter is working properly. For example, test a second slide using a different probe (but labelled with the same fluorochrome) and observe it under the same microscope as the originally tested probe.
- Try increasing the denaturation temperature or increasing the denaturation time. However, if other FISH probes are working as required, be cautious with altering processes.

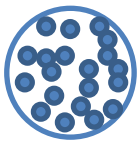
Table 1. Troubleshooting G-banding

Problem	**Possible Cause**	**Action**
Bands too faint 	Over banded	Reduce trypsin time Increase stain time
Bands too dark 	Stain time too long Not enough trypsin	Reduce stain time Increase trypsin time
Too pale	Pale batch of stain Stain time too short	Increase stain time Increase concentration of stain

		
Too dark 	Stain time too long Stain too old	Reduce stain time Make up a fresh batch of Leishman's solution Dilute stain
Grey all over 	Not enough trypsin	Increase trypsin time
Fuzzy 	Underaged / over banded	Age longer / reduce trypsin time

Table 2. FISH slide assessment guide

Slide appearance	Quality	Interpretation
	Thick	Slides that are too thick and densely populated will cause probe signals to interfere with each other. If this occurs then the slide maker will need to thin out the material by adding more

		fixative. Generally, a 13 mm circumference coverslip is suitable.
	Ideal	Interphases are nicely spaced apart with a good number of figures but not overlapping each other. Generally, a 13 mm circumference coverslip is suitable.
	Thin or nothing	Interphases are sparse with very few under one plain of view. The slide maker would need to add another drop (maybe 2) of fixed material. Generally, a 16 mm circumference coverslip is suitable.

5 ACKNOWLEDGMENTS

This work was supported by the grant from the UK Regenerative Medicine Platform, MRC reference MR/R015724/1.

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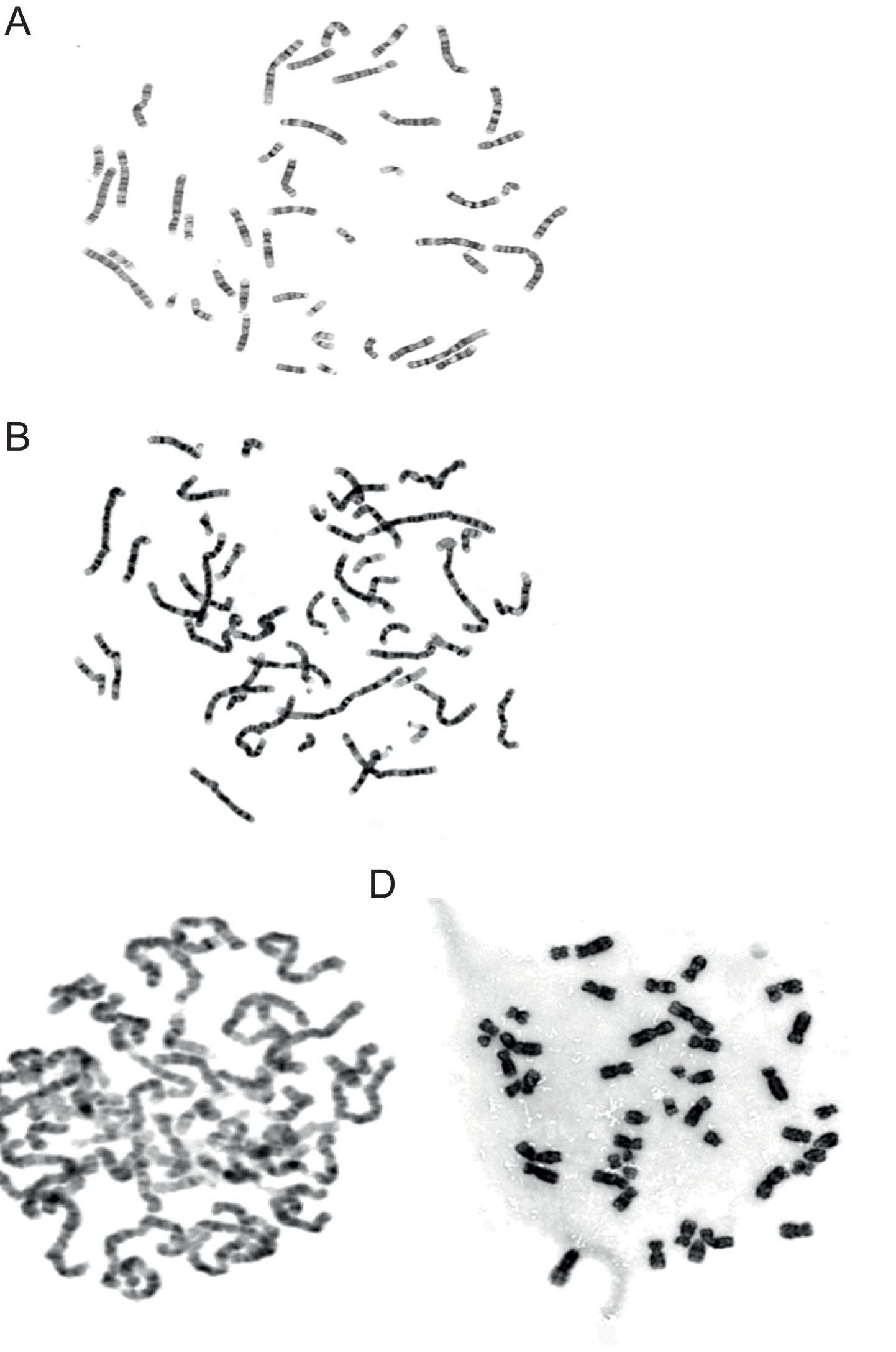
Figures:

Figure 1. The types of chromosome spreading and G-banding quality. A) Good spread and banding: chromosomes are spread out well, with little overlap of individual chromosomes. Chromosome boundaries are well defined and no cytoplasm is visible. B) OK spread and banding: chromosomes are spread out well, but there is a lot of overlap of individual chromosomes. Cytoplasm is not observed. C) Poor spreading: Chromosomes are ill-defined and overlapping, making the analysis impossible. D) Cytoplasmic encapsulated metaphase: Chromosomes are not well defined, G-banding is impaired by the cytoplasmic debris and analysis is impossible.

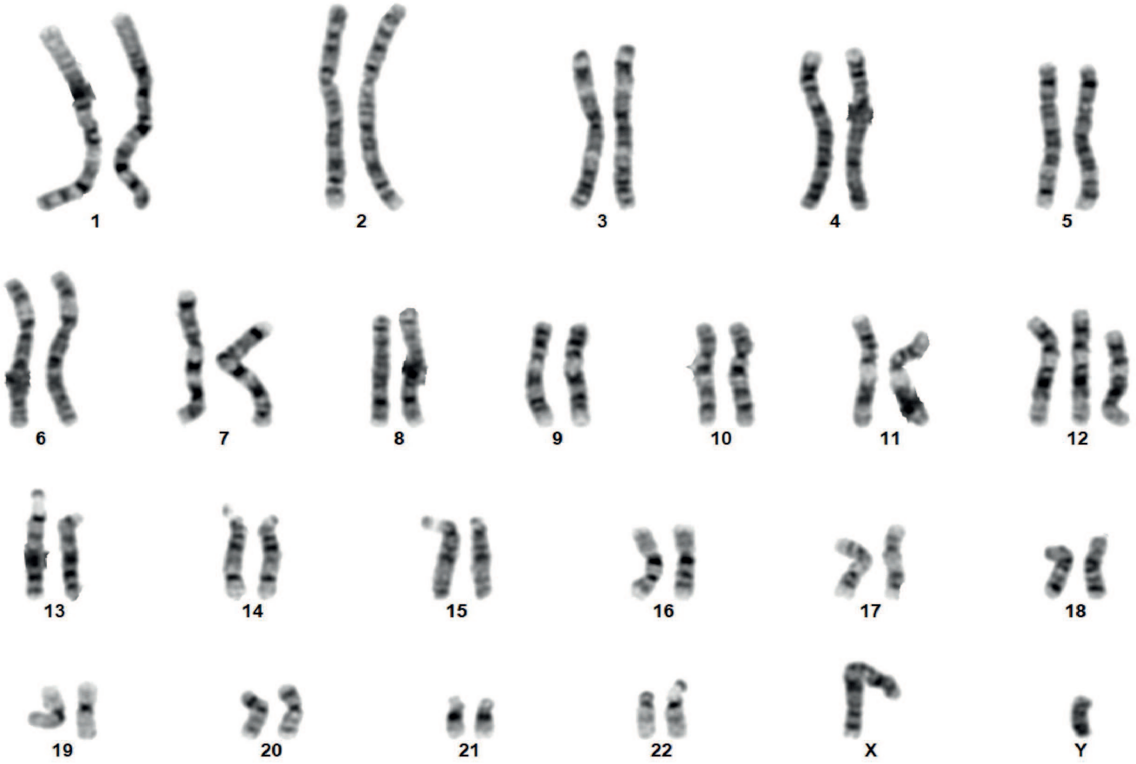
Figure 2. Frequently encountered genetic aberrations in hPSCs. A) A karyogram of a primed hPSC with a gain of chromosome 12 (trisomy 12). B) A metaphase of a polyploid naïve hPSC.

Figure 3. Examples of FISH preparation quality of A) metaphase and B) interphase FISH. Ai) A good quality metaphase FISH with the probe fluorescents signals visible as neat, bright foci, unobscured by unspecific background. Aii) A poor quality metaphase FISH with damaged, likely overly denatured, chromosomes. Bi) A good quality interphase FISH with the probe signals clearly identifiable as bright single foci. Bii) A poor quality interphase FISH

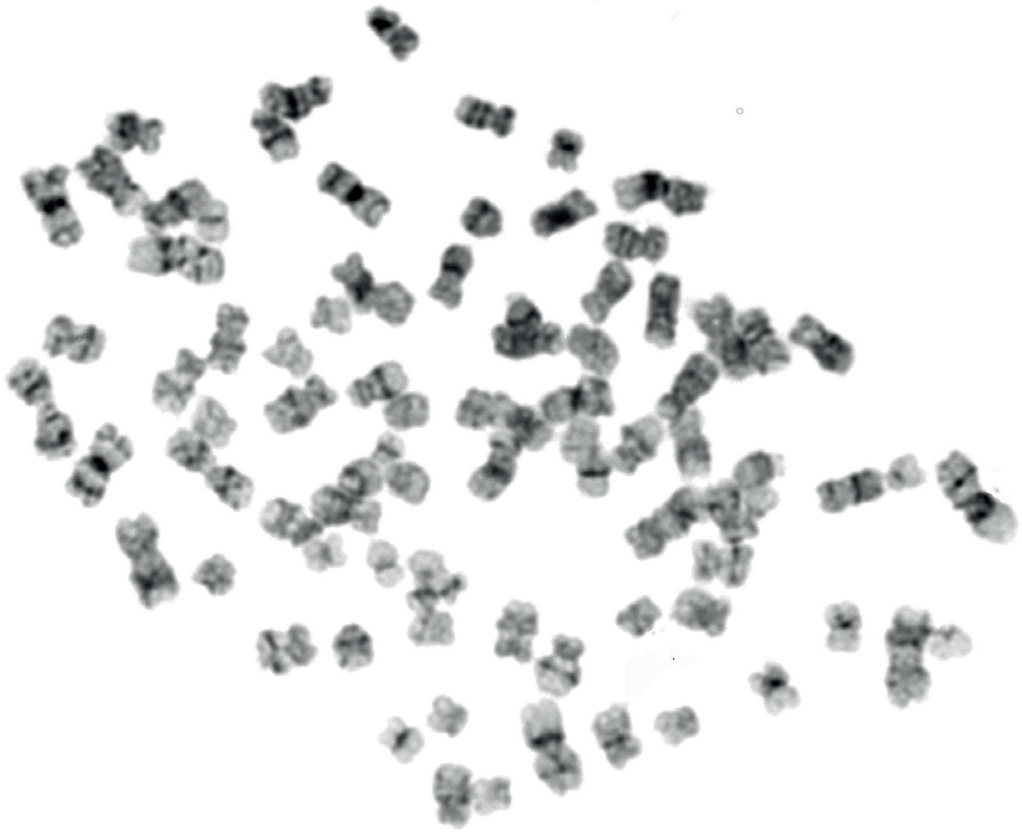
with the faint and speckled probe fluorescents signals. Nuclei are counterstained with DAPI (blue).


A
B
C
D

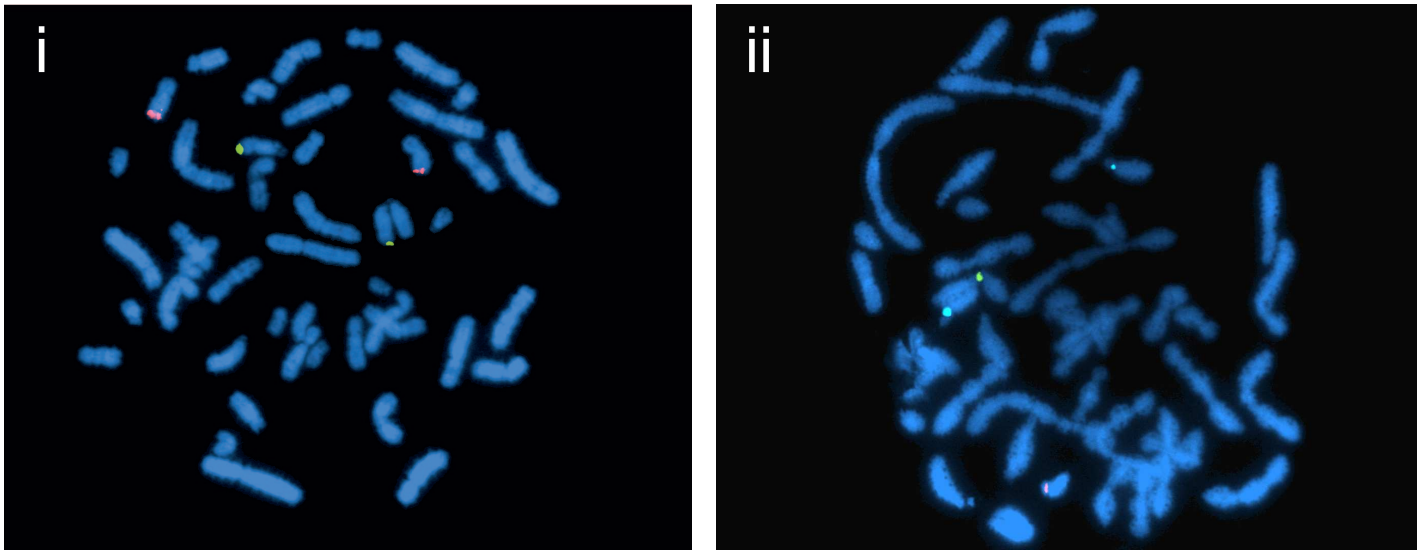
A



B



A



B