FiberPrep®

DNA Extraction Kit

DNA Extraction Protocol

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Reference: IFU-M&S-13-c

GENOMIC VISION

CONTENT

1. INTRODUCTION

This FiberPrep® DNA Extraction kit has been specifically designed to provide high quality DNA solutions for Molecular Combing. Molecular Combing is Genomic Vision's proprietary technology used for many applications and particularly for studying:

- DNA replication: physical characteristics and kinetic parameters of DNA replication can be studied by monitoring the incorporation of modified nucleotides in growing cells.
- Large genetic rearrangements: by use of specific labeled probes, large rearrangements (from 1 kb to several Mb) can be detected. This includes duplications, deletions, contractions or expansions and CNVs generally, as well as balanced translocations, inversions, etc.

The DNA is then stretched onto silanized coverslips (COV-002, Genomic Vision, Paris, France) using the FiberComb® Molecular Combing System (MCS-001, Genomic Vision, Paris, France). Several hundreds of single DNA molecules are stretched in a parallel way and with a constant stretching factor, which enables the correspondence between a physical measure and the DNA length (1 µm = 2 kb). The hybridization and detection steps are followed by scanning with the FiberVision® (SCN-001, Genomic Vision, Paris, France) or FiberVisionS® (SCN-002, Genomic Vision, Paris, France) automated scanner and analysis with the dedicated FiberStudio® software (FSE-LSR-P1, Genomic Vision, Paris, France).

2. INTENDED USE & PRINCIPLE OF PROCEDURE

The FiberPrep® DNA Extraction kit is intended for the extraction of DNA from fresh blood samples, purified peripheral blood mononuclear cells (PBMC) or cultured eukaryotic cell lines for Molecular Combing applications. This kit is designed to purify and store high molecular weight DNA (average size: 400 kb), by protecting them from mechanical stress. After embedding cells in an agarose plug, proteins are digested by a proteinase and cell membranes are solubilized by a surfactant under inhibition of DNase activity. The treated DNA plugs are then ready to be used for the preparation of a high molecular weight DNA solution by agarase digestion.

3. STORAGE CONDITIONS

The complete kit is divided into two parts according to the conditions of storage (+2°C to +8°C and -25°C to -10°C). Each element is necessary for the entire process of extraction.

4. PRODUCT USE LIMITATIONS

For general laboratory use.

5. KIT REAGENTS AND STORAGE CONDITIONS

- The +2/+8°C part of the FiberPrep® DNA Extraction kit (Cooler box) contains:

Buffers	Volume in the kit	Storage temperature	Condition after opening
Buffer 2 (Plug Buffer)	7 mL	+2°C to +8°C	Stable for 1 month after opening Do not melt and refrigerate more than 10 times
Buffer 3 (Proteinase Buffer)	25 mL	+2°C to +8°C	N/A
Buffer 4 (Washing Buffer)	110 mL	+2°C to +8°C	N/A
Buffer 5 (Storage Buffer)	280 mL	+2°C to +8°C	N/A
Buffer 6 (Combing Buffer)	250 mL	+2°C to +8°C	Stable 6 months after opening
Component 3 (Proteinase)	3 mL	+2°C to +8°C	N/A

- The -20°C part of the FiberPrep® DNA Extraction kit contains:

Buffers	Volume in the kit	Storage temperature	Condition after opening
Buffer 1 (Suspension Buffer) *	7 mL	-25°C to -10°C	Storage at +2-8°C and stable for 1 month after opening
Component 6 (Agarase)	165 µL	-25°C to -10°C	N/A

* Buffer 1 is only used for extraction of DNA from cultured cells. If you extract your DNA from blood, please replace Buffer 1 by standard cell culture grade PBS (Phosphate-Buffered Saline).

The FiberPrep® DNA Extraction Kit contains reagents to perform 100 extractions, preparing the plugs independently or by several (by 2, 3 or 10 plugs at the same time for example).

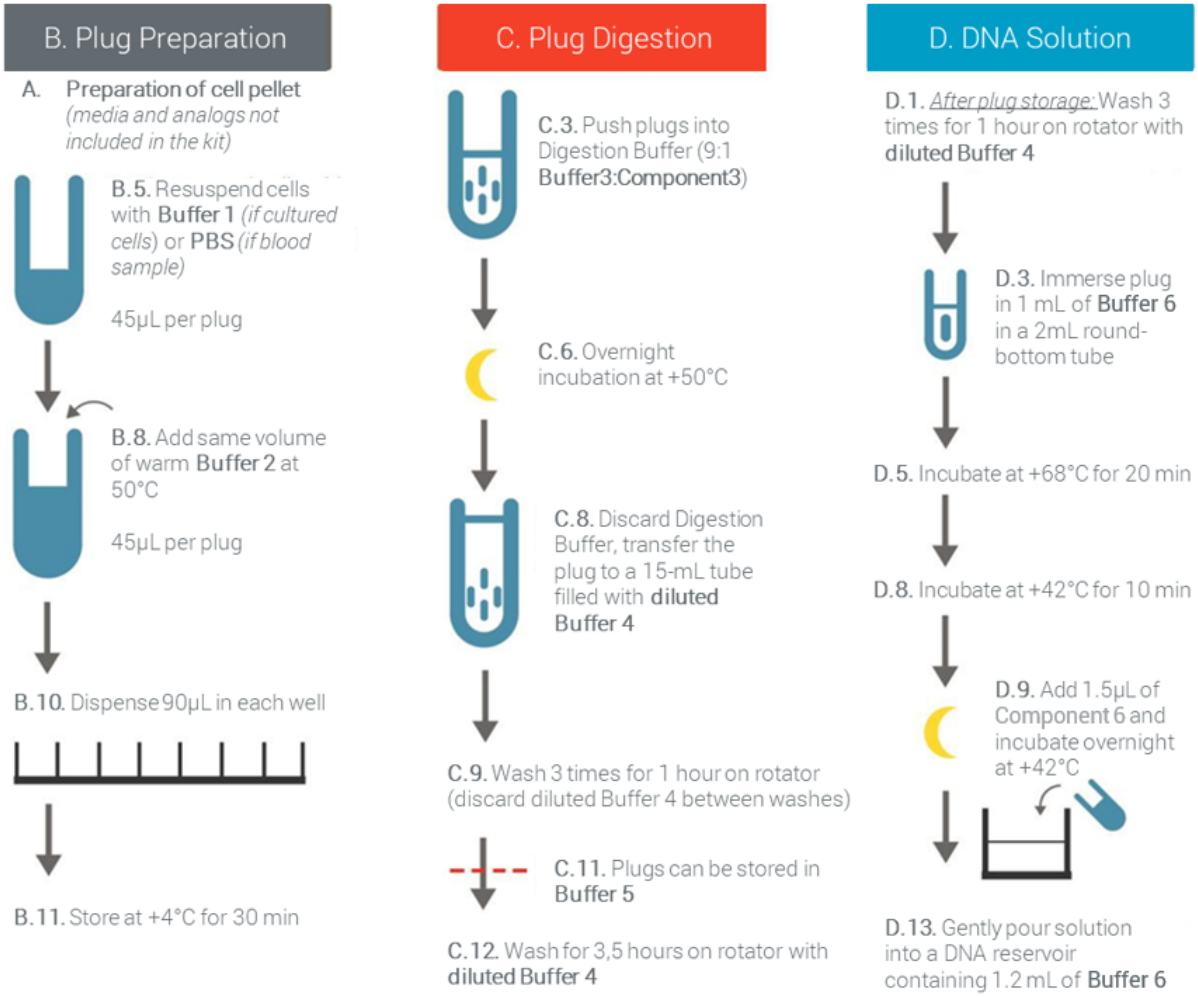
6. SAFETY PRECAUTIONS AND WARNINGS

- Wear a suitable lab coat, disposable gloves and protective goggles when handling reagents and samples. Thoroughly wash hands before and after handling them.
- Do not pipette by mouth.
- Samples and reagents of human origin as well as contaminated material and products must be discarded in a contaminated residue container.
- If liquid containing human material is spilled, clean the affected area with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.
- For more information, please consult the appropriate material safety data sheets (MSDS) on our website: www.genomicvision.com.

7. ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED

Materials	Supplier	Reference
FiberComb® Molecular Combing System	Genomic Vision	MCS-001
Coverslips	Genomic Vision	COV-002
Disposable Reservoir	Genomic Vision	RES-001
Gel plug Mold	BioRad	1703713
Test tube rotator	Any	-
Waterbath	Any	-
Heating Block +42°C	Any	-
Cell culture CO_2 incubator	Any	-
Counting Chamber	Any	-
Falcon tube 15mL and 50mL	Any	-
2mL Round-bottom centrifuge tube	Any	-
Cell culture flask	Any	-
Centrifuge	Any	-
PBS	Any	-
Support for coverslip	Any	-
Screened caps	BioRad	1703711
YOYO-1	ThermoFisher	Y3601
Mounting medium	Vectashield or Slowfade or Prolong	-

8. ASSAY PROCEDURE



8.1 Preparation of cells (Step A)

Before starting cell manipulation:

- Set two water baths at +68°C and +50°C.
- Melt the Buffer 2 of FiberPrep® kit at +68°C for 10 min. *Check that the gel is completely melted*. Homogenize the melted solution by inverting the tube and keep it at +50°C until use.

For *cultured cells*, harvest your cells according to cell lines' specifications. The cells can be adherent or in suspension.

Respect safety measures associated with the type of cells you are manipulating. Cells should be considered intact until Digestion treatment of cells is completed.

For *blood samples*, best results are obtained with white blood cells purified by either red blood cell lysis or Ficoll® gradient centrifugation. Blood should be fresh or have a maximum storage time of 5 days at 4°C. Tested anticoagulants are EDTA, ACD. This procedure is not validated for extraction from frozen blood.

8.2 Plug preparation: Embedding cells into agarose plugs (Step B)

Caution: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis

The number of cells to be embedded in a plug depends on the downstream application:

- For replication studies using approximately 10,000 cells / plug is recommended.
- For physical cartography (hybridization) applications, typically 500,000 – 1,000,000 cells / plug is recommended.
- Optimal cell number has to be decided by users for their own applications.

1. Count cell number of your cell suspension with a Kovas® slide and a microscope or another equivalent.
2. Centrifuge the appropriate quantity of cells suspension at 160g for 5 min at room temperature.

Tip: Due to pipetting loss and/or errors, we recommend to perform the experiment with a dead volume corresponding to 0,5 extra plug until plugging.

3. Decant the supernatant of cell suspension gently. Remove remaining supernatant by pipette as much as possible.
4. Calculate the appropriate volume of **Buffer 1** (45 µL is needed for 1 plug). Thaw 30 minutes at ambient temperature the appropriate quantity of Buffer 1 or use tubes of Buffer 1 already opened for less than 1 month and stored at +4°C.

Tip: Continue to count 0,5 extra plug because of pipetting loss and/or errors.

Example: For the preparation of 5 plugs, use 247.5 µL (= 5.5 x 45 µL) of the cell suspension.

5. Completely suspend the cell pellet with the appropriate volume of **Buffer 1** (for cultured cells) or **PBS** (for cells extracted from blood).

Caution: Do not refreeze the tube of Buffer 1, store it at +4°C for maximum 1 month.

6. Homogenize well by pipetting (10 times up and down).

Caution: It is important that any clumps of cells have been removed prior to embedding cells into the agarose plug.

7. Warm the cell suspension to +50°C for 10 seconds.
8. Mix the suspension with the same volume of **melted Buffer 2** (identical to the volume previously calculated for Buffer 1) kept at +50°C as indicated in step A.
9. Homogenize the solution well at +50°C by pipetting (10 times up and down).

Caution: Make sure that the cell/Buffer 2 mix is homogeneous and no clumps are visible.

10. Immediately dispense the mixture quickly into DNA plug molds. The volume of one well is approximately 90 µL. Keep the solution in the water bath while dispensing and do not hesitate to homogenize between each distribution by pipetting up and down.

Caution: Do not allow bubbles to form.

11. Set the DNA plug mold horizontally at +4°C (in a box to prevent the agarose gel from desiccating) for 30 min.

Tip: If the plugs are not well solidified or look too soft, incubate them 15 min longer at +4°C.

8.3 Protein digestion treatment of plugs (Step C)

1. Prepare 250 µL of complete **Protease digestion buffer** (**Buffer 3** + **Component 3**, 9:1 volume) per plug. Plugs from the same condition may be incubated together. Use a 15 mL-tube for up to 3 plugs, or a 50 mL-tube for more than 3 plugs and up to 10 plugs.

⚠ *Caution: This mix cannot be prepared in advance*

Example: For 10 plugs, add 250 µL of Component 3 to 2250 µL of Buffer 3.

2. Push the solidified plug out of the mold into the **Digestion Buffer** using a plastic plunger.

Tip: Place the plugs on the surface of the tube and slide them up to the digestion buffer to prevent them from being damaged by falling violently into the solution.

3. Warm up the tube containing the plug(s) at +50°C. Since the tubes contain small volumes of solution, make sure the tubes are held vertically in the water bath.
4. After 30 min at +50°C, gently swirl the tubes to homogenize the solution.
5. Keep the tubes at +50°C overnight (16-18 hours).
6. The following day, dilute enough **Buffer 4** in a 1:100 proportion with nuclease-free water for the following four washing steps (or three washing steps if you store the plug). If possible, autoclave **diluted Buffer 4** in advance or use only recently diluted Buffer. Do not store it.
   According to the number of plugs, use 15 mL-tube (for up to 3 plugs) or 50mL-tube (for up to 10 plugs). If you plan to store or not the plug, prepare different amount of buffer 4 in a 1:100 proportion.

Example:

Condition	*With storage in Buffer 5 (3 washes)*		*Without storage in Buffer 5 (4 washes)*	
Type of tube	*15 mL-tube*	*50 mL-tube*	*15 mL-tube*	*50 mL-tube*
Buffer 4 volume	*0,5 mL*	*1,7 mL*	*0,7 mL*	*2,3 mL*
Nuclease-free water	*49,5 mL*	*168,5 mL*	*69,3 mL*	*227,7 mL*
Total volume	*50 mL*	*170 mL*	*70 mL*	*230 mL*

7. Transfer DNA plugs to appropriate tube(s) filled with **diluted Buffer 4** (max 3 plugs/15 mL-tube and 10 plugs/50 mL-tube) with a spatula. Be careful when manipulating the plugs.

Tip: Make sure there is a small air bubble in the tube, to allow a soft flow of liquid. If the bubble is too big, it may damage the plugs, if it is too small, it may get stuck in the tube. Check the movement of the bubble after putting the tube on the test tube rotator.

8. Wash the plugs for 1 hour on a test tube rotator.
9. Change the **diluted Buffer 4** and wash again for 1 hour with rotation. Repeat twice (in total 3 washes).

Tip: Screened caps can be useful for efficiently recovering the plug and removing the washing buffer.

10. If you store the plugs, transfer the plugs in:
    - 1 mL of Buffer 5 in a 2 mL-round-bottom microtube if handling one plug,
    - 5 mL of Buffer 5 in a 15 mL-tube for up to 3 plugs,
    - 10 mL of Buffer 5 in a 50 mL-tube for a maximum of 10 plugs.

Plugs can be stored for 1 year at +4 °C in **Buffer 5**.

Tip: We recommend to store them independently in 2mL-round bottom microtube for easier recovery in the event that future use is made by 1 plug.

OR

11. If you use this DNA plug immediately for DNA solution preparation, change once again the **diluted Buffer 4** in the tube and wash for 3.5 hours with rotation. Then go directly to step 2 of the next section (8.4. Preparation of DNA solution from DNA plug).

8.4 Preparation of DNA solution from DNA plug (Step D)

If DNA plug is freshly prepared, go to step 2.

1. If DNA plug has been stored in Buffer 5, wash the plug in 15 mL of **diluted Buffer 4** (1:100 proportion Buffer 4: Nuclease-free water) in a 15 mL or 50 mL-tube for 60 min at room temperature, on a test tube rotator. Repeat twice (in total 3 washes).
2. Using a spatula, transfer one washed plug to a 2 mL-round-bottom microtube.
3. Add 1 mL of **Buffer 6** to the microtube.
4. Check that the plug is completely immersed. If not, tap gently the tube on the bench until the plug drops to the bottom of the tube.
5. Set the tube on a fixed microtube holder and confirm that the microtube is not moved by current of water bath. Incubate at +68°C for 20 min.

⚠ *Caution: From this step on, manipulate DNA solutions with care, as DNA is no longer protected from mechanical shearing. Avoid any shaking, vibration, shock, etc.*

6. During this incubation, equilibrate a heating block at +42°C.
7. After 20 min at +68°C, quickly transfer the microtube to the heating block at +42°C. DO NOT leave the microtube at room temperature before the transfert at +42°C.
8. Incubate the microtube at +42°C for 10 min**. Beware to avoid any shaking**.
9. Add 1.5 µL of **component 6** to the microtube. Hold the **component 6** in the micropipette tip for 10 seconds before injecting it into the DNA solution. In fact, the component 6 is cold while the buffer 6 is hot, leaving it for a few seconds at room temperature will prevent a thermal shock. Gently inject the **component 6** at the surface of the solution. Do NOT mix the solution, as this would bring to DNA breakage. Let the component 6 diffuse spontaneously.

⚠ *Caution: Move the component 6 out of the freezer with a cold block and return it to -20 ° C as soon as possible.*

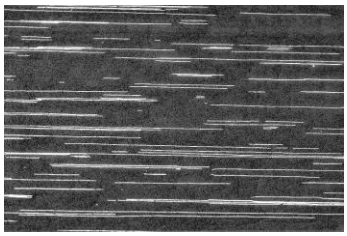
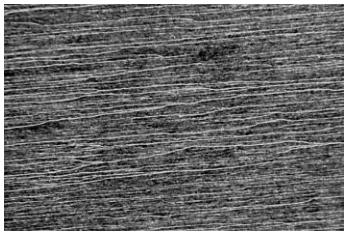
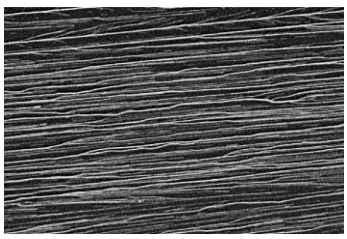
10. Incubate the microtube overnight (16-18 hours) at +42°C. Avoid any shaking.

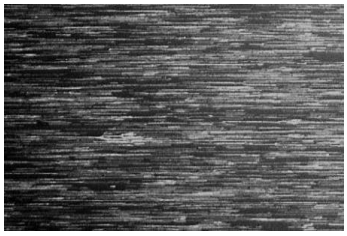
11. The following day, remove the microtube from the heating block.
12. Add 1.2 mL of **Buffer 6** into a Disposable DNA Reservoir in advance.
13. Gently pour the DNA solution into the disposable DNA reservoir. Fill the reservoir with additional **Buffer 6** if necessary (up to a few millimeters below the top). After 30 min at room temperature, the DNA solution can be combed on a silanized coverslip with the FiberComb® Molecular Combing System. You can store the DNA solution up to 1 week at +4°C, let the solution at least 30 min at room temperature before its combing.

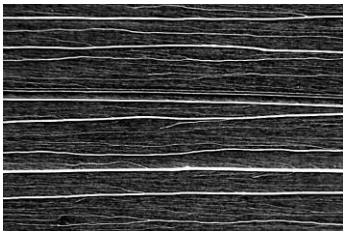
Note: Before proceeding to immunodetection of your replication tracks, the quality of DNA extraction can be checked under a fluorescence microscope.

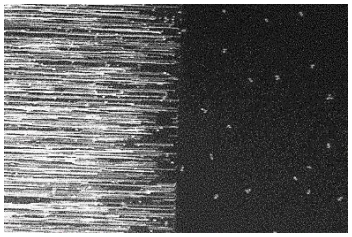
- *Bake the combed coverslip for 3 min on hotplate at 125°C*
- *Mount the coverslips with Yoyo1 (recommended concentration 1:10 000 with Prolong or SlowFade) to stain combed dsDNA. Once mounted, the coverslip cannot be reused. Perform the immunodetection steps on another combed coverslip.*
- *The imaging and data analysis can be automatically processed with the FiberVision® or FiberVisionS® scanner, using the FiberCheck algorithm, or with the EasyScan service provided by Genomic Vision. A manual imaging can also be performed using a regular fluorescent microscope.*

9. TROUBLE SHOOTING GUIDE

Problem	Possible Cause	Suggestion
1. Low combing density = Low DNA concentration in the solution (low density of combed DNA on silanized coverslip) 	1.a) Low cell count	Make sure the cells were completely resuspended and homogenized when adding Buffer 1, and later on Buffer 2, by pipetting up and down at least 10 times.
	1.b) Loss of Proteinase activity	Check the Proteinase storage temperature and make sure to avoid any contamination sources when handling the Component 6. Use a new Component 6 aliquot.
	1.c) pH degradation of Buffer 6	Measure the pH of Buffer 6. If it is not 5.5, change Buffer 6.
2. High combing density = Too high DNA concentration in the solution (wavy or entangled molecules) 	2.a) DNA solution is too concentrated in the reservoir	Diluting solution with Buffer 6 is a possibility but may result in fiber breakage.
	2.b) High cell count	If initial material / sample is still available: decrease the number of cells used during plug preparation (prepare DNA plug with lower concentration of cells). If no initial material is available, use half or part of a plug.
3. Linearity of DNA fibers = Combed DNA shows wavy molecules on silanized coverslip 	3.a) Combing DNA solution while vibrating environment	Avoid any source of vibration close to MCS while combing.
	3.b) Insufficient plug washing	Washing time may be too short: try again with extended washing time.
	3.c) Problem (localized or not) on combing substrate (silanized coverslip)	Change substrate batch or surface.
	3.d) Mechanical stress after combing (during labeling process)	Comb a new silanized coverslip from the same solution and re-start the labeling process.
	3.e) pH degradation of Buffer 6	Cf. 1.c)

Problem	Possible Cause	Suggestion
4. Short DNA fibers = Length of combed DNA on silanized coverslip is too short (DNA breakage)	4.a) Sharp handling of the DNA solution	From step 5 of section D (Preparation of DNA solution from DNA plug) the DNA is no longer protected from mechanical stress. Avoid any shaking, vibration or sharp movements when handling the DNA solutions.
	4.b) Insufficient plug washing	Washing timing may be too short: try again with extended washing time.
	4.c) Degraded starting sample material (cells)	If the starting material is not in optimal conditions (using old or frozen blood, for example), it is not possible to obtain a high quality DNA solution.
	4.d) Contamination with DNase	Avoid contamination sources and/or change buffers.
	4.e) Water bath temperature (> 68°C)	Check temperature of your water baths with a validated thermometer.
	4.f) Time of water bath (> 20 min)	Check duration of water baths steps.
	4.g) Vibrating source around DNA	cf. 4.a)
	4.h) Buffer 4 has been diluted with contaminated water and/or stored in diluted form.	Prepare a fresh solution of Buffer 4 and autoclave it. If it is not possible, always use recently diluted Buffer 4. Do not store it.
	4.i) Buffer 2 is too old or damaged and the gel can no longer protect the DNA from mechanical breaks.	Use a new bottle of Buffer 2 or a new kit.
	4.j) DNA is shortening during real time observation -> not inherently broken but breakage is induced during long exposure under microscope light.	Lower lamp intensity / power or reduce exposition time. Baking your coverslip in an oven at 60°C for 4 hr will reduce photobreaking. *-> Note: the DNA solution quality is not the issue and can still be used for another combing for direct observation or hybridization (DNA molecules are not broken in solution).*

Problem	Possible Cause	Suggestion
5. DNA is not individualized = presence of very bright fiber bundles, network… 	5.a) Non-efficient digestion of proteins during digestion step	Check the storage temperature and if the protocol has been followed carefully, or change Digestion buffer and/or Buffer 2. *-> Caution: storage temperature of the enzymes is critical (Trypsin, Component 3 and Component 6 are thermosensitive)*
	5.b) Homogenization of cells during plug preparation is insufficient (cell aggregates)	Repeat plug preparation with extended mixing, check when cell counting that cells are isolated correctly and evenly distributed.
	5.c) Activity of Trypsin is degraded	Change Buffer 1 tube. Avoid multiple thawing/freezing of Buffer 1.
	5.d) Problem during cell harvesting (adherent cells)	Cf. 5.b)
	5.e) Defect localized on substrate	Change silanized coverslip or batch.
	5.f) DNA is not well distributed / homogenized in combing buffer solution / Suspension is not achieved	Repeat combing a day (or a few more days) later.
	5.g) DNA is too concentrated in solution	Cf. 2)
6. Bad stability of DNA solution = Density (or length) is lowered over time / No DNA after 24 hr of solution storage	6.a) Contamination of DNA solution with DNase or other DNA damaging agent (ex. metal ion…)	Change or autoclave materials that may be contaminated (spatula, tubes, pipette tips, …)
	6.b) Vibration / shock of DNA solution during storage	Pay attention to avoid vibrations during storage and handling.
	6.c) Aging / natural degradation of DNA in solution	Recommendation is to comb DNA solution as soon as possible, storage in disposable reservoirs with Buffer 6 should not extend more than 6 days. It is recommended to store plugs rather than DNA solution of a specific sample, as plugs are more stable.
	6.d) Contamination of solution during combing	If possible, do all necessary combing in one session. We recommend to store plugs rather than combing the solution of a specific sample.

Problem	Possible Cause	Suggestion
7. Contrast of combed DNA = Low signal/noise ratio, signals are very low intensity and high background.	7.a) Premature aging of silanized coverslip or silanized coverslip defect	Repeat combing changing silanized coverslip.
	7.b) Mounting medium is not homogenized enough	Repeat combing and observation after homogenizing mounting medium (vortex).
	7.c) Mounting medium is too concentrated in marker (YOYO-1)	Prepare a new mounting medium making sure to have an adequate concentration.
8. Residues in solution = Visible colored or white residues (pellet or fiber) remain in the genomic DNA solution	8.a) Entangled DNA molecules	You may try to reach the residue using the tip of a pipette (autoclaved), and take it out of the solution. However, resulting DNA solution can be broken or still entangled.
	8.b) DNA aggregates due to a bad homogenization of cells during plug preparation	When possible, repeat plug preparation with extended mixing and check when cell counting that cells are isolated correctly and evenly distributed.
9. Defect on silanized coverslip not linked to DNA solution (white or black structure or stains, crystals, scratch, …) 	9.a) If defects are visible on combed coverslip, check if defects are also present at the limit of combing (where the surface has not been dipped into DNA solution) : if so, it may be defects coming from silanized coverslip	This is not a defect coming from the DNA solution. Try to comb again using another silanized coverslip or silanized coverslip batch.
	9.b) Scratch performed during manipulation	Pay attention to avoid touching the surface you want to observe. If your silanized coverslip falls down on the bench, you may try to lift it using a small piece of tape or tweezers, avoiding any friction against material. Best option remains to comb another silanized coverslip.
10. Colored Buffer 1 -> normal colour is pink/reddish but it may turn yellow/purple depending on pH concentration or degradation	10.a) pH of solution is not correct	Do not use the Buffer 1. Use another aliquot.
	10.b) Buffer 1 has been left to ambient temperature instead of 4°C after first thawing of -20°C aliquot	Do not use the Buffer 1. Use another aliquot.
11. White precipitate in Buffer 6 (salt crystals, …)	11.a) Water has evaporated from the Buffer 6, or temperature of DNA solution was excessively cooled (below 4°C) and precipitates have formed	Concentration in salts may not be correct. Do not use the Buffer 6. Use another aliquot.
12. Buffer 6 has turned slightly yellow	12.a) Inappropriate storage condition (Buffer 6 should be stored in refrigerator at 4°C)	Do not use the Buffer 6. Use another aliquot.

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Quality Control

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