# Studying physical chromatin interactions in plants using Chromosome Conformation Capture (3C)

Louwers, M.; Splinter, E.; van Driel, R.; de Laat, W.; Stam, M.

**DOI**
10.1038/nprot.2009.113

**Publication date**
2009

**Document Version**
Author accepted manuscript

**Published in**
Nature Protocols

# Studying physical chromatin interactions in plants using Chromosome Conformation Capture (3C)

Marieke Louwers[1,2], Erik Splinter[3], Roel van Driel[1], Wouter de Laat[3], Maike Stam[1,*]

*[1]Swammerdam Institute for Life Sciences, Universiteit van Amsterdam, Science Park 904 1098 XH Amsterdam, The Netherlands. [2]Current address: CropDesign N.V., Technologiepark 3, 9052 Zwijnaarde, Belgium. [3]Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.*

* Correspondence: m.e.stam@uva.nl (e-mail), +31-20-5257655 (phone); +31-20-5257935 (fax).

**Abstract**

Gene regulation in higher eukaryotes frequently involves physical interactions between genomic sequence elements tens of kilobases apart on the same chromosome, but can also entail interactions between different chromosomes. Chromosome Conformation Capture (3C) is a powerful tool to identify such interactions. 3C technology is based on formaldehyde crosslinking of chromatin, followed by restriction digestion and intramolecular ligation. Quantitative detection of ligation products by PCR (qPCR; not discussed in this Protocol) provides insight into the interaction frequencies between chromosomal fragments and thereby the spatial organization of a genomic region. Detailed 3C protocols have been published for yeast and mammals. These protocols can however not simply be transferred to plant tissues. In this paper we provide a maize-specific 3C protocol and present a general strategy to systematically optimize the protocol for other plants. Once the technique and appropriate controls are established, the 3C procedure (including qPCR) can be completed in 5-7 days.

## 'INTRODUCTION'

Over the last few years it has become clear that long-range physical interactions between distant chromosomal regions play an important role in gene regulation. Regulatory elements are often located ten to hundreds of kilobases (kb) away from the target promoter they affect. Using 3C, a powerful technique to identify long-range chromatin interactions, it has been shown that the regulation of gene expression by such distantly located chromosomal regions occurs through physical interactions [1,2]. Interestingly, chromosomal interactions not only play a role in gene activation, but also in X chromosome inactivation [3,4] and the maintenance of transcriptionally silent chromatin states [5]. Although it is becoming clear that gene regulation by long-range interactions is widespread, little is still known about this level of chromosome organization, especially for plants.

### Methods to investigate chromatin interactions

Three different techniques are currently being used to study chromosomal interactions: Fluorescent *in situ* hybridization (FISH) [3,6,7], RNA-Tagging and Recovering of Associated Proteins (RNA-TRAP) [7] and Chromosome Conformation Capture (3C) [1,8]. Each technique is based on a different principle and uses a distinct method. Therefore these techniques can be used to complement and validate each other.

FISH utilizes fluorescently labeled DNA probes to detect genes or chromosomes by fluorescence microscopy. FISH can for instance be used to observe chromosome abnormalities, or to map molecular markers. When probing two regions in the genome, the FISH signals can be used to study the frequency of co-localization of two chromosomal regions [3,4,9]. FISH has the advantage that cell-to-cell variation can easily be investigated. The microscope, however, has a limited spatial resolution. As a result, co-localization is not necessarily associated with physical interactions.

The RNA-TRAP technique is based on targeted *in situ* hybridization and is used to study physical interactions at actively transcribed sequences [7,10,11]. With RNA-TRAP, horseradish peroxidase (HRP) is targeted to nascent RNA transcripts, followed by HRP-catalyzed biotin deposition on nearby chromatin. The biotinylated chromatin is isolated by streptavidin affinity chromatography, followed by DNA isolation and quantification by PCR. This method relies on the presence of nascent RNA at a chromosomal locus and is therefore not generally applicable.

The 3C technology (Fig. 1a) captures physical interactions between chromosomal regions by crosslinking proteins to proteins, and proteins to DNA with formaldehyde. The crosslinked chromatin is subsequently digested by restriction enzymes, followed by intramolecular ligation and reversal of the crosslinks overnight. The DNA is purified and evaluated by PCR analysis. The resulting data indicate the frequency with which DNA fragments of interest are crosslinked to other fragments. Chromosomal regions are only crosslinked together when they are in close proximity. Importantly, 3C can be used whether a gene is on or off and is consequently widely applicable to study chromatin interactions [1,8,12,13].

Up until recently, only the FISH method has been applied to plant tissue. RNA-TRAP has only been used for mammalian systems, and 3C for yeast, Drosophila and mammals [1,5,8,12,13]. The existing protocols for the last two methods cannot simply be transferred to plant tissues due to the special properties and unique chemical composition of plants. In this paper we provide a maize-specific 3C protocol that we recently developed and successfully applied [14]. Moreover, we present a general strategy to systematically optimize the protocol for other plants. This paper will therefore be of interest to scientists that want to use 3C in plant research, but can also be of interest to those that want to develop or optimize a 3C protocol for other organisms. The presented protocol is based on previously published 3C protocols [12,13], but contains several plant-specific adjustments. The major adjustments are 1) the fixation of nuclei within plant tissue, 2) the nuclei isolation from plant tissue, 3) the need to work with freshly isolated material and nuclei, 4) the inactivation of endogenous nucleases, and 5) the

optimization of the isolation of ligation products. The adjustments are discussed in detail below.

The purified ligation products are analyzed by PCR analysis, either semi-quantitatively, using conventional PCR amplification followed by agarose gel-separation [13], or by using TaqMan®-based quantitative PCR (qPCR) [12,15]. We consider qPCR using TaqMan® chemistry to be the best method to accurately measure crosslinking frequencies. Accurate quantification requires that measurements are taken in the linear range of amplification. This requirement is fulfilled by the qPCR method, but challenging to achieve when using conventional PCR. In the latter method, ethidium bromide (EtBr)-stained fragments on an agarose gel are quantified. The main drawbacks of quantification via conventional PCR include saturation of the PCR amplification and/or of the EtBr-staining. For the quantification of ligation products by TaqMan® qPCR, and also for the subsequent data analysis, we have successfully implemented [14] the same protocol as described in Hagege et al. (2007). This procedural information is therefore not included in this paper.

The 3C technique is suitable to identify chromatin interactions within a single genomic region of a few hundred kb. To allow an unbiased, genome-wide search for chromatin interactions, more recently, different 3C-based techniques (4C, 5C) have been developed that make use of micro-array analyses or high-throughput sequencing (reviewed in ref. 16). 4C techniques (3C-on-chip or circular 3C) identify chromatin interactions between one particular sequence region of interest and the rest of the genome [17,18], while the 5C technique (3C-carbon copy) allows the detection of chromatin interactions between multiple sequence regions of interest and the rest of the genome [19]. ChIP-loop and ChIA-PET are yet other adaptations to the 3C technique [2,20,21]. These methods combine 3C with chromatin immunoprecipitation (ChIP) and Paired-End Tag sequencing [22] to identify long-range chromatin interactions that involve a particular protein complex. For more details on the above mentioned 3C-based techniques we recommend the papers cited.

**Experimental design**

Figure 1b displays the outline and timing of a typical 3C experiment. To obtain high quality 3C data, a careful experimental design and critical data analysis are crucial. Every single step of the protocol has to work optimally to avoid misinterpretation of the data. When using the protocol for the first time, we recommend evaluating the quantity and morphology of the nuclei (see Box 1). Furthermore, it is crucial to always check the digestion and ligation efficiency before performing TaqMan® qPCR quantifications of the ligation products. To

apply the provided protocol to other plants than maize, the following steps may have to be optimized: the fixation of plant tissue, the isolation of nuclei, the restriction digestion and the purification of ligation products. These steps, together with ideas for optimization are described in detail below. To be able to interpret the 3C data obtained, various experimental controls are absolutely essential. The most important ones are a control for PCR efficiency and a control to correct for differences in amount and quality of the template. These controls are excellently described in Dekker et al. (2006) and recapitulated below.

**Fixation of plant tissue**

To ensure efficient crosslinking of plant tissue, the fixation conditions should be optimal [13]. In 3C protocols, DNA and proteins are crosslinked with formaldehyde to stabilize the chromatin conformation. Cells in culture, the starting point of most mammalian 3C protocols, are relatively easy to fix. This is not true for cells in plant tissue, the usual starting material in plant research. Plant tissue has a waxy surface and spongy air-filled mesophyll, and every cell is surrounded by a thick cell wall, hampering the crosslinking agent to penetrate the tissue and enter the cells. A way to circumvent this problem is to first isolate nuclei, followed by a fixation step. We however noticed that the yield and quality of nuclei are much higher when plant tissue is treated with formaldehyde prior to nuclei isolation. We recommend the use of soft plant tissue, and if possible, tissue that is relatively rich in unexpanded cells. Such tissue will facilitate the penetration of formaldehyde, shortening the fixation time, and will provide a better yield and purity of nuclei. Furthermore, the use of tissue samples with a relatively homogeneous texture will avoid differences in fixation efficiency within the tissue. To ensure optimal penetration of the crosslinking agent, the tissue should be cut into small pieces before fixation, and crosslinked under vacuum (see step 5 of PROCEDURE and TROUBLESHOOTING). To further increase the penetration of the fixative, we recommend switching on and off the vacuum several times. Plant tissue that has been successfully penetrated by formaldehyde becomes translucent, except for the plant veins. The crosslinking conditions reported in this paper (step 4-6 of PROCEDURE) work well for inner stem and husk tissue of maize (see step 1 of PROCEDURE for details on these tissues), but should be tested and possibly optimized when using different maize tissues or tissue of another plant. To this end, both the fixation time as well as the concentration of the crosslinking agent can be varied. When deciding to use a different nuclei isolation buffer than listed in this paper, it is important to realize that ingredients of a buffer can interfere with the crosslinking agent (see also TROUBLESHOOTING). For example, buffers containing Tris hamper fixation by

formaldehyde. Tris contains reactive amine, causing crosslinking of formaldehyde to Tris, leaving less formaldehyde available to crosslink DNA and proteins. Furthermore, some nuclei isolation protocols use buffers that do not contain the correct cations to stabilize the chromatin (see TROUBLESHOOTING).

**Nuclei isolation**

For a 3C experiment, approximately $10^7$ nuclei are needed, and importantly, the nuclei must be of good quality. Surrounding debris must be eliminated as much as possible. As with the crosslinking step, the plant's waxy cuticle and the thick cell walls hamper the efficient isolation of sufficient, qualitatively good nuclei in which the chromatin conformation is preserved. Therefore, the isolation of nuclei suitable for 3C requires a plant-specific approach. The protocol presented here yields sufficient nuclei of a quality suitable as starting material for 3C (see Box 1, Fig. 2 and TROUBLESHOOTING). Importantly, the nuclei isolation protocol also yields sufficient and good quality nuclei from *Arabidopsis* leaf tissue (Fig. 2a), indicating that the method presented is suitable for a range of plants. Too many centrifugation steps or careless handling of nuclei can lead to insufficient and/or broken nuclei. Nuclei are fragile. We recommend limiting the number of centrifugations steps as much as possible and to handle the nuclei with great care.

**Restriction digestion in intact nuclei**

Genomic sequence elements such as promoters and enhancers need to be physically separated by restriction digestion to allow the analysis of relative crosslinking frequencies. To obtain meaningful 3C data, the efficiency of restriction digestion should be at least 60-70%, but preferentially more than 80%. Partial digestion leads to reduction in the number of restriction ends available for ligation, which diminishes the efficiency of the 3C experiment and, importantly, can introduce biases in the 3C analysis. To achieve efficient digestion in maize nuclei, it is necessary to always use freshly harvested tissue and proceed through the entire 3C protocol once the tissue is harvested. The storage of tissue or nuclei at -80 ºC results in poor digestion efficiencies, possibly due to deterioration of nuclei or to less accessible chromatin.

The digestion is performed on intact, crosslinked nuclei. In order for the restriction enzyme to have sufficient access to the target chromatin, the nuclei have to be permeabilized. This is achieved by incubating the nuclei with the detergent SDS. After this treatment, the nuclei are distorted, no longer intact and often tend to aggregate (See Fig. 2b). Aggregation may hamper the accessibility to restriction enzymes. To counteract aggregation of the nuclei,

they are shaken during the incubation steps. The SDS treatment does not only permeabilize the nuclei, it is also supposed to inactivate any non-crosslinked proteins, in particular nucleases, preventing DNA degradation. For maize nuclei, however, the standard SDS treatment at 37 ºC appeared not sufficient to remove all nucleases, resulting in DNA decay (see Fig. 3a and M.S., unpublished results). To inactivate remaining nucleases, we have introduced an additional heating step at 65 ºC (see step 22 of PROCEDURE, ANTICIPATED RESULTS and Fig. 3a).

Only a limited number of restriction enzymes efficiently digest DNA in the context of crosslinked nuclei. Three 6-base cutters have been reported to work well: *Bgl*II, *Eco*RI and *Hind*III [13], and we achieved near-complete digestion with *Bam*HI after lowering the SDS concentration used to permeabilize the nuclei (see TROUBLESHOOTING). Six-base cutters are preferred when analyzing loci up to several hundreds of kilobases. To analyze loci up to only 20 kb, or to fine-map interactions observed with 6-base cutters, 4-base cutters such as *Dpn*II and *Nla*III may be used [12]. The enzyme of choice preferably generates sticky ends, facilitating the ligation step. The enzyme should in addition be insensitive to cytosine methylation, avoiding incomplete digestion as a result of DNA methylation. When setting up or optimizing the 3C protocol for a specific tissue, organism or restriction enzyme, we recommend to always check the digestion efficiency by DNA blot analysis (see ANTICIPATED RESULTS and Fig. 4) or by qPCR across restriction sites (see Box 2 in ref. 12). The digestion efficiency may differ depending on the brand of restriction enyzme used. Once optimal digestion conditions have been established and actual 3C experiments are being performed, we recommend testing the digestion efficiency by agarose gel electrophoresis for every experiment. For more information, see Box 2 and Figure 3.

**Purification of ligation products**

The digested DNA is ligated at a low DNA concentration, favouring intramolecular over intermolecular ligation. After the ligation, the crosslinks have to be reversed and the DNA purified. The DNA purification involves an ethanol precipitation (steps 40-42). For maize DNA, the precipitation proved to be very inefficient. Adding a carrier, such as glycogen, to the reaction greatly improved the DNA recovery. Yields may also differ depending on the brand of polypropylene tubes used; we use BD Biosciences Falcon tubes. Once the DNA has been precipitated and washed, the pellet needs to be homogenously dissolved. For maize DNA, dissolving the pellet by incubating at 55 ºC for 20 min, as done in many other protocols, was not effective. We recommend dissolving for several hours at room temperature

(18-22 $^{o}$C), followed by one night at 4 ºC (step 47). Should any undissolved particles remain after overnight incubation, gently spin the sample and take the clear supernatant.

**Controls required for 3C**

Two important experimental controls that need to be taken along in order to interpret the 3C data correctly, are a control for PCR efficiency and an endogenous control to correct for differences in amount and quality of the template [1,23]. To be able to compare crosslinking frequencies that are measured for different DNA restriction fragments, a control must be included to normalize the differences in PCR efficiency between different primer sets. To this end, a control template is required that contains all possible ligation products of the genomic regions of interest, in equimolar amounts. This template can be prepared by restriction digestion and subsequent random ligation of a BAC clone covering the sequences of interest [1]. Alternatively, equimolar amounts of the DNA fragments spanning each of the restriction sites to be analyzed can be mixed, digested and ligated. Be aware however, that this latter method is more error prone as it assumes every DNA fragment in the mix to be of similar quality.

A second crucial control that must be taken along is an endogenous control. This control corrects for differences in quality and quantity of genomic template between different samples and between different tissues. For the endogenous control, crosslinking frequencies are measured between two restriction fragments of a genomic locus that is unlinked to the locus of interest. A control locus is expected to adopt the same spatial configuration in all tissues to be examined by 3C. A similar expression level in the tissues of interest is a good indication this is indeed the case. Furthermore, to avoid the potential influence of chromosome folding, the interaction frequencies at a control locus must be measured over a distance smaller than 8kb. To ensure that the resulting PCR fragment is not the consequence of incomplete restriction digestion, primers must be designed unidirectionally, or the chosen fragments should be separated by at least one kb. Ideally, the fragments to be analyzed are separated by three or four sites. The qPCR signal of the endogenous control should be in the range of the signals obtained for the locus of interest. The presence of multiple restriction sites at the endogenous control locus allows to choose the ligation product that results in the required signal level. In our study we have used the maize *S-adenosyl-methionine decarboxylase* gene (*Sam*; Fig. 5). *Sam* was identified to fulfill the required criteria [14]. It showed a similar expression level in our tissues of interest, maize inner stem and husk, and within about 2 kb, five *Bgl*II sites were present (*Bgl*II is used for our 3C experiments). A primer set annealing at the outer two sites resulted in a qPCR signal level equal to that of the

locus of interest and serves as our endogenous control. If using other maize tissues, we recommend testing the *Sam* transcript levels for these tissues. For more details on controls we refer to existing protocols and papers [1,12-15,23].

**Box 1: Determination of quality and quantity of nuclei**

Simonis *et al.* (2007) pointed out that frequent interactions between neighbouring fragments on a linear chromosome are captured in less than 1 in 500 cells, while long-range *in cis* and *in trans* interactions are captured in only 1 in 10,000 or even 1 in 100,000 cells. Therefore, it is crucial to isolate a relatively large quantity (~10 million) of nuclei that are furthermore of satisfactory quality. This box provides three protocols (A-C) how to monitor the intactness and quantity of the nuclei at various steps in the 3C procedure.

Protocol A 'TIMING' 15 min

(i) Take a 2 µl aliquot from the nuclei suspensions in steps 12 and 14.

(ii) Stain the nuclei with DAPI (2 µg $ml^{-1}$ final) or Methyl Green-Pyronin (MGP).

(iii) Check the yield and nuclear morphology under a fluorescence (DAPI) or light (MGP) microscope. A large quantity of round, intact nuclei should be visible while the amount of debris should be fairly low (see Fig. 2a).

'TROUBLESHOOTING'

(iv) Proceed with steps 13 (aliquot step 12) or 15 (aliquot step 14) when the quality and quantity of nuclei appears satisfactory.

Protocol B 'TIMING' 15 min

(i) Take a 3 µl aliquot from the nuclei suspension in step 16 and stain the nuclei with DAPI or MGP.

(ii) Dilute the nuclei suspension 10x in nuclei isolation buffer.

(iii) Count the nuclei under a fluorescence (DAPI) or light (MGP) microscope using a counting chamber. The nuclei should be intact and round.

(iv) Calculate the amount of nuclei per µl, and use 1 x $10^7$ nuclei to proceed with step 18 from the main procedure.

Protocol C 'TIMING' 15 min

(i) To check if the nuclei are sufficiently permeabilized, take a 2 µl aliquot after the SDS incubation in step 22 and stain it with DAPI or MGP.

(ii) Check the nuclear morphology using a fluorescence (DAPI) or light (MGP) microscope. The nuclei should now be seriously distorted, aggregated and (partially) broken down (see Fig. 2b).

(iii) Proceed with step 23 from the main procedure.

**Box 2: Determination of digestion efficiency 'TIMING' 2 days**

The DNA digestion efficiency should be above 60-70%, but ideally >80%. Therefore, the efficiency should be carefully analyzed in every 3C experiment, even when the optimal restriction conditions have been determined (see ANTICIPATED RESULTS). This analysis can be done in parallel with steps 34-48 of the main procedure. Samples with low digestion efficiency should be discarded.

(i) Add 5 µl proteinase K to the 300 ul aliquot taken in step 32.

(ii) Reverse the crosslinks overnight at 65 ºC.

(iii) Add 5µl RNase A and incubate for 30-45 min at 37 ºC.

(iv) Add 310 µl Phenol-Chloroform-Isoamylalcohol and mix well by rigorously shaking the tubes by hand.

'CRITICAL' The Phenol-Chloroform-Isoamylalcohol solution should be at room temperature (18-22 $^{o}$C) to allow adequate phase separation. 'CAUTION' Phenol-Chloroform-Isoamylalcohol is toxic and corrosive. Use only in the fumehood.

(v) Centrifuge for 10 min at 16,000*g* at room temperature (18-22 $^{o}$C).

(vi) Transfer the aqueous phase to a new 1.5 ml Eppendorf tube.

'CAUTION' Dispose of Phenol-Chloroform-Isoamylalcohol waste according to the existing regulations.

(vii) Add 31 µl 2M NaOAC pH5.6, 600 µl 100% EtOH (v/v) and 1 µl glycogen (20 mg/ml) and mix well by inverting the tube several times.

(viii) Store at -80 ºC for at least 1 h.

'PAUSE POINT' The tube can be stored at -80 ºC overnight.

(ix) Centrifuge the frozen tube for 45 min at 16,000*g* at 4 ºC.

(x) Discard the supernatant and wash the pellets with 500 µl cold 70% (v/v) EtOH.

(xi) Centrifuge for 5 min at 16,000*g* at 4 ºC.

(xii) Remove as much liquid as possible and air-dry the pellets for 3-5 minutes.

(xiii) Dissolve the pellet in 30 µl 10 mM Tris pH 7.5 by incubation at room temperature (18-22 $^{o}$C) for several hours, followed by overnight incubation at 4 ºC.

(xiv) The sample is now ready for agarose gel electrophoresis. Proceed with step 48 of the main procedure (see also ANTICIPATED RESULTS and Fig. 3).

**‘MATERIALS’**

**‘REAGENTS’**

- Nuclei isolation buffer (see REAGENT SETUP)
- Hepes (Sigma, cat. no. H4034)
- Sucrose (Fluka, cat. no. 84100)
- $MgCl_2$ Hexahydrate (Fluka, cat. no. 63072)
- KCl (Analar, cat. no. 101985)
- Glycerol (Merck, 87%, cat. no. 1.04094)
- Triton X-100 (20% (v/v); Sigma, cat. no. T8787) ‘CRITICAL’ Triton stock and work solutions are light-sensitive and should be kept in the dark; work solutions should be relatively fresh (maximum 1-2 months old).
- Phenylmethylsulfonyl Fluoride, PMSF (Sigma, cat. no. P7626; see REAGENT SETUP) ‘CAUTION’ Harmful if inhaled or absorbed by skin. The PMSF waste should be disposed of according to the regulations.
- 2-mercapto-ethanol (Merck, cat. no. 1.15433) ‘CAUTION’ Toxic and dangerous for the environment. Harmful if inhaled or absorbed by skin. The 2-mercapto-ethanol waste should be disposed of according to the regulations.
- Formaldehyde solution, 4% (w/v). Prepare from paraformaldehyde (ICN Biomedicals, Inc., cat. no. 150146; see REAGENT SETUP) ‘CAUTION’ Toxic and corrosive. Dangerous if inhaled, absorbed through skin or swallowed.
- Glycine, 2 M (ICN Biomedicals, Inc., cat. no. 194825)
- Liquid nitrogen
- Complete protease inhibitor tablets (Roche, cat. no. 11697498001; see REAGENT SETUP)
- Miracloth (Calbiochem, cat. no. 475855)
- Sefar Nitex filter, poresize 50 μm (Sefar Inc.) See http://www.sefar.com for more information.
- 4’,6-Diamidine-2-phenylindole dihydrichloride, DAPI (Roche, cat. no. 10236276001) and/or Methyl Green-Pyronin (MGP) ‘CRITICAL’ DAPI and Methyl Green-Pyronin solutions are light-sensitive and should always be kept in the dark.
- Restriction enzyme buffer (for *Bgl*II SuRE/Cut Buffer M; Roche, cat. no. 11417983001)

- Sodium Dodecyl Sulphate (SDS 20% (w/v); US Biological, cat. no. 85010) 'CAUTION' Harmful if swallowed or inhaled. Irritating to skin, eyes and respiratory tract.
- High concentration restriction enzyme (for *Bgl*II, Roche, cat. no. 11175068001)
- 10 x ligation buffer (see REAGENT SETUP)
- Trizma base (Sigma, cat. no. T6066)
- Dithiotreitol, 1 M (DTT; dissolved in 0.01 M NaOAc; Sigma, cat. no. D9779) 'CAUTION' Irritating to skin and eyes. Harmful if swallowed or inhaled.
- Adenosine triphosphate, ATP (Sigma, cat. no. A-2383)
- Proteinase K (Roche, cat. no. 3115879001), 10 mg $ml^{-1}$
- T4 DNA ligase, high concentration
- Ribonuclease A (RNase A; Roche, cat. no. 10109169001), 10 mg $ml^{-1}$
- Phenol, buffered with TE pH 8 (Sigma, cat. no. P4557) 'CAUTION' Toxic and corrosive. Dangerous if inhaled, absorbed through skin or swallowed. Use phenol only in the fumehood. Phenol waste should be disposed of according to the regulations.
- Chloroform (Merck, cat. no. 1.02445) 'CAUTION' Toxic and harmful for environment. Dangerous if inhaled, absorbed through skin or swallowed. Use chloroform in the fumehood. Chloroform waste should be disposed of according to the regulations.
- Isoamylalcohol (ICN Biochemicals, cat. no. 1.4059) 'CAUTION' Harmful if swallowed, irritating to respiratory system and skin. Isoamylalcohol waste should be disposed of according to the regulations.
- Phenol-Chloroform-Isoamylalcohol, 25:24:1 (see REAGENT SETUP).
- Sodium Acetate (Anhydrous), 2 M pH 5.6, (NaOAc; Sigma, cat. no. S2889)
- Ethanol (Nedalco, 96% (v/v); cat. no. 104.000.0020E)
- Glycogen, 20 mg $ml^{-1}$ (Fermentas, cat. no. R0561)
- Tris-HCl 10 mM pH 7.5 and 7.8 at 25 ºC
- Agarose (Roche, cat. no. 11388991001)
- Ethidium Bromide, 10 mg $ml^{-1}$ (EtBr; Biorad, cat. no. 161-0433) 'CAUTION' EtBr is a mutagen. Always wear gloves when handling gels and solutions containing EtBr. Dispose of EtBr-containing gels and solutions appropriately.

**'EQUIPMENT'**

- Speedvac (Savant Speedvac Plus SC110A; see EQUIPMENT SETUP; brand not critical) or exiccator
- Sorvall centrifuge with SS-34 rotor (Centrifuge RC5C Meyvis; brand not critical)
- Refrigerated microcentrifuge (Eppendorf, model 5417R; brand not critical)
- Eppendorf Thermomixer (brand not critical)
- Water bath (brand not critical)
- Centrifuge for 50 ml Falcon tubes (Heraeus Labofuge 400R; brand not critical)
- Electrophoresis equipment (Embi Tec, RunOne; Blue casting stand EP-1019; brand not critical)
- Nalgene Oak ridge centrifuge tubes PPCO (Nalge Company, cat. no. 3119-0050)
- Falcon tubes BD Biosciences (cat. no. 352070 – 5098512; brand critical)
- Eppendorf tubes (1.5 and 2 ml; Safe-Lock)
- Funnel (80 mm diameter)
- Surgical knife and blades
- Petri dish (15 cm diameter)
- Polystyrene (~25 mm thick)
- Forceps
- Mortar and Pestle

**'REAGENT SETUP'**

**Nuclei isolation buffer** 20 mM Hepes pH 8; 250 mM Sucrose; 1 mM $MgCl_2$; 5 mM KCl; 40% (v/v) glycerol; 0.25% (v/v) Triton X-100; 0.1 mM PMSF; 0.1% (v/v) 2-mercapto-ethanol. Add PMSF and 2-mercapto-ethanol immediately before use.

'CAUTION' Both PMSF and 2-mercapto-ethanol are harmful if inhaled or absorbed by the skin.

**PMSF** Prepare a 100 mM stock solution in isopropanol. Make aliquots in 1.5 ml Eppendorf tubes and store at -20 ºC.

**Formaldehyde solution, 4% (w/v)** Dissolve 4 gram of paraformaldehyde in 100 ml nuclei isolation buffer. Set to pH 9 to decompose the polymer into formaldehyde. Heat to 65 ºC to aid in dissolving. Let the solution cool down, adjust to pH 7-7.5 and store aliquots at -80 ºC.

Thaw only once. 'CAUTION' Toxic and corrosive. Dangerous if inhaled, absorbed through skin or swallowed. Formaldehyde waste should be disposed of according to the regulations.

**Protease inhibitor solution** Dissolve 1 complete protease inhibitor tablet (Roche) in 2 ml $H_2O$. The obtained solution can be stored at 4 ºC if used within 2 weeks. Store at -20 ºC for longer storage (up to 3 months).

**10 x ligation buffer** 300 mM Tris-HCl (pH 7.8 at 25 ºC); 100 mM $MgCl_2$; 100 mM DTT, 10 mM ATP. Always use freshly made ATP for the preparation of the ligation buffer. Store aliquots of the buffer at -20 ºC. Thaw the buffer only once. The composition of this ligation buffer is identical to the one provided by Promega (cat. no. C1263). Due to the large amounts required, we prepared the ligation buffer ourselves.

**Phenol-Chloroform-Isoamylalcohol 25:24:1** Mix Chloroform and Isoamylalcohol in a 24:1 ratio and combine this solution in a 1:1 ratio with TE-buffered Phenol. Allow the mixture to equilibrate overnight at 4 ºC. 'CRITICAL' The Phenol-Chloroform-Isoamylalcohol solution has to be used at room temperature (18-22 $^{o}$C). Therefore, place the solution at room temperature the day before use.

'CAUTION' Phenol and Chloroform are toxic, Isoamylalcohol is harmful. All three are dangerous if inhaled, absorbed through skin or swallowed.

**'EQUIPMENT SETUP'**

**Speedvac** For fixation under vacuum conditions, remove the rotor from the Speedvac centrifuge and place a box or rack that can hold 50 ml tubes into the centrifuge.

**'PROCEDURE'**

This protocol is optimized for maize tissue; some adaptations may be required when this protocol is used for other plant species.

**Preparation of plant tissue 'TIMING' 15 min**

**1** Use approximately 1.5 grams of plant material. This protocol works well with maize husk (leaves surrounding the cob) and inner stem tissue (young sheaths and leaves surrounding the shoot meristem of 5 to 6-week-old plants), and yields 10-12 x $10^6$ nuclei. For husk tissue, use $1/4^{th}$ of the leaves. Use only soft leaves; discard the tough, outer leaves. In our hands, the simultaneous processing of a maximum of 4 samples works out best.

‘CRITICAL STEP’ Always use freshly harvested tissue. Do not use tissue stored at -80 ºC; the use of such tissue hampers efficient restriction digestion (step 25).

**2** Use a surgical knife to cut the tissue in small pieces (~ 1 $cm^3$) in a large petri dish placed on ice.

**3** Transfer the tissue to a 50 ml Falcon tube and add 15 ml ice-cold nuclei isolation buffer.

**Formaldehyde crosslinking ‘TIMING’ 70 min**

**4** Add 15 ml 4% (w/v) formaldehyde solution (final: 2%) and mix by inverting.

‘CAUTION” Formaldehyde is dangerous if inhaled, absorbed through skin or swallowed.

**5** Remove the cap and clog the tube with a piece of polystyrene to prevent the tissue from spilling out during vacuum infiltration. The piece of polystyrene should have the diameter of the tube and contain a small incision through which air (but not tissue) can escape. Place the cap loosely on the tube and place the tube in the speedvac. Vacuum infiltrate for 1 h at room temperature (18-22 $^{o}$C). At the start, switch the vacuum on and off for 2-3 times. After one hour, the material should be slightly translucent.

‘CRITICAL STEP’ Fixing plant tissue under vacuum helps the crosslinking agent to enter the cells, especially when switching the vacuum on and off for a few times.

‘TROUBLESHOOTING’

**6** Remove the piece of polystyrene with forceps and add 1/16 volume cold 2 M Glycine (final: 0.125 M). Mix by inverting and place the cap loosely on the tube. Incubate for 5 min under vacuum at room temperature (18-22 $^{o}$C) to stop the crosslinking.

**Isolation of intact maize nuclei ‘TIMING’ 1-2 h**

**7** Pour the liquid out of the Falcon tube and rinse the tissue in the tube three times with MilliQ water. ‘CAUTION’ PMSF, 2-mercapto-ethanol and formaldehyde are toxic and harmful, dispose the waste according to the appropriate regulations.

**8** Dry the tissue between paper towels.

**9** Transfer the tissue to a chilled, clean mortar and grind it with liquid nitrogen until the tissue is pulverized into a fine powder.

‘CRITICAL STEP’ Once the tissue is frozen in liquid nitrogen, prevent thawing of the tissue.

‘TROUBLESHOOTING’

**10** Add protease inhibitor solution to ice-cold isolation buffer (1 ml/25 ml).

**11** Add 10 ml ice-cold nuclei isolation buffer, containing protease inhibitors, to the ground tissue. Gently mix the isolation buffer with the pulverized tissue and allow the frozen sample to thaw.

**12** Place a Nalgene Oakridge tube on ice, put a funnel on top. Place a piece of Miracloth on top of a piece of Sefar Nitex mesh (both diameter 120 mm), and fold a filter. Place the filter in the funnel. Once the sample is liquefied, pour it through the filter (The Sefar Nitex mesh can be rinsed and re-used). Take a 2 µl aliquot of the filtered nuclei suspension and analyze the sample as described in Box 1, protocol A. Once the 3C method is set up, this test is no longer required (see ANTICIPATED RESULTS).

'CRITICAL STEP' Work on ice to keep the nuclei intact.

'TROUBLESHOOTING'

**13** Spin the nuclei suspension for 15 min at 3000*g* at 4 ºC (Sorvall centrifuge, rotor SS-34).

'CRITICAL STEP' The isolated nuclei are quite fragile. Handle them with care, keep them cold at all times and do not spin faster than described.

**14** Gently pour or pipette off the supernatant and dissolve the pellet in 1 ml ice-cold nuclei isolation buffer containing protein inhibitors. The pellet should dissolve easily by gently swirling the tube. If not, gently pipette up and down. Take a 2 µl aliquot of the nuclei suspension and analyze the sample as described in Box 1, protocol A. Once the 3C method is set up, this test is no longer required (see ANTICIPATED RESULTS).

'TROUBLESHOOTING'

**15** Transfer the nuclei suspension to an Eppendorf tube and centrifuge 5 min at 1900*g* at 4 ºC in an Eppendorf centrifuge.

**16** Pipette off the supernatant and gently take up the pellet in 1 ml ice-cold nuclei isolation buffer containing protein inhibitors. Careful, the pellet can be loose. Take a 3 µl aliquot of the nuclei suspension and analyze the sample as described in Box 1, protocol B. Use 1 x $10^7$ nuclei for a 3C experiment.

**17** Centrifuge the nuclei 5 min at 1900*g* at 4 ºC in an Eppendorf centrifuge and pipette off the supernatant.

**DNA digestion 'TIMING' 18-20 h**

**18** Resuspend the nuclei in 400 µl 1.2 x restriction enzyme buffer.

'CRITICAL STEP' Traces of the plant nuclei isolation buffer can hamper the digestion. To increase the restriction digestion efficiency, the nuclei are washed once in restriction buffer.

Use freshly isolated nuclei. In our hands, freezing and long-term storage of nuclei at -80 $^{o}$C systematically leads to partial restriction digestion.

**19** Centrifuge for 5 min at 1900*g* at 4 ºC in an Eppendorf centrifuge.

**20** Remove the supernatant and resuspend the pellet in 500 µl 1.2 x restriction buffer. If necessary, gently pipette up and down.

**21** Add 7.5 µl 20% (w/v) SDS (final: 0.3% SDS).

**22** Shake at 900 r.p.m. for 40 min at 65 ºC, followed by 20 min shaking at 37 ºC. Take a 2 µl aliquot of the nuclei suspension and analyze the sample as described in Box 1, protocol C. Once 3C is set up, this is no longer required.

'CRITICAL STEP' The incubation step at 65 ºC in the presence of SDS is necessary to inactivate endogenous nucleases (see INTRODUCTION, ANTICIPATED RESULTS and Fig. 3a).

**23** Add 50 µl 20% (v/v) Triton X-100 (final: 2% TX-100).

'CRITICAL STEP' SDS hampers the restriction digestion and is therefore sequestered by Triton X-100.

**24** Shake at 900 r.p.m. for 60 min at 37 ºC.

**25** Add 400 U of the selected restriction enzyme and incubate overnight at 37 ºC while shaking at 900 r.p.m.

When setting up and/or optimizing the protocol, take 1/3$^{th}$ of the sample after the overnight incubation to check the digestion efficiency by DNA blot analysis (see ANTICIPATED RESULTS and Fig. 4).

'TROUBLESHOOTING'

**Intramolecular ligation 'TIMING' 7-8 h + overnight step**

**26** To inactivate the restriction enzyme, add 40 µl 20% (w/v) SDS (final: 1.6% SDS).

**27** Incubate for 20-25 min at 65 ºC while shaking at 900 r.p.m.

**28** Transfer the sample to a 50 ml Falcon tube.

**29** Add 7 ml 1x ligation buffer (700 µl 10x Promega Ligation buffer plus 6.3 ml sterile MilliQ).

**30** Add 375 µl 20% (v/v) Triton X-100 (final: 1% Triton X-100).

'CRITICAL STEP' SDS hampers the ligation and is therefore sequestered by addition of Triton X-100.

**31** Incubate for 1 h at 37 ºC.

**32** To check the digestion efficiency, transfer a 300 µl aliquot to a 1.5 ml Safe-Lock Eppendorf tube and analyze the sample as described in Box 2. This analysis can be done in parallel with steps 34-48 of the main procedure.

'TROUBLESHOOTING'

**33** Add 100 U highly concentrated DNA ligase and incubate for 5 h at 16 ºC, followed by 45 min at room temperature (18-22 $^{o}$C).

'CRITICAL STEP' The brand of ligation buffer proved to be critical in our hands.

**34** Add 30 µl proteinase K (total 300 µg).

**35** Incubate the sample at 65 ºC overnight to reverse the crosslinks.

**DNA purification 'TIMING' 4-5 h + overnight step**

**36** Add 30 µl RNase A (total 300 µg) and incubate 30-45 min at 37 ºC.

**37** Add 10 ml Phenol-Chloroform-Isoamylalcohol and mix well by rigorously shaking the tube by hand.

'CRITICAL' The Phenol-Chloroform-Isoamylalcohol solution should be at room temperature (18-22 $^{o}$C) to allow adequate separation of the phases.

'TROUBLESHOOTING'

**38** Centrifuge for 10 min at 4500*g* at room temperature (18-22 $^{o}$C).

**39** Transfer the aqueous phase to a new Falcon tube.

'CRITICAL STEP' The brand of Falcon tubes proved to be critical in our hands.

'CAUTION' Dispose of Phenol-Chloroform-Isoamylalcohol waste according to the existing regulations.

**40** Add 7 ml sterile MilliQ, 1400 µl 2 M NaOAC pH 5.6, 40 µl glycogen and 24 ml 96% (v/v) EtOH and mix well by inverting the tube several times.

'CRITICAL STEP' A high DTT concentration hampers DNA precipitation, therefore the concentration of DTT is diluted prior to the precipitation step. Furthermore, adding a carrier such as glycogen greatly improves the DNA precipitation.

**41** Store at -80 ºC for at least 2 h.

'PAUSE POINT' The tube can be stored at -80 ºC overnight.

**42** Centrifuge the frozen tube for 60 min at 4500*g* at 4 ºC.

**43** Discard the supernatant and wash the pellet with 10 ml cold 70% (v/v) EtOH.

'TROUBLESHOOTING'

**44** Centrifuge for 15 min at 4500*g* at 4 ºC.

**45** Pipette off as much liquid as possible and air-dry the pellet for 3-5 minutes.

**46** Add 150 µl 10 mM Tris pH 7.5 to the pellet.

**47** To dissolve the DNA, incubate the pellet at room temperature (18-22 $^{o}$C) for several hours followed by overnight incubation at 4 ºC.

'CRITICAL STEP' It is crucial that the DNA is completely dissolved before proceeding with the qPCR analysis. In case any undissolved particles remain after overnight incubation, gently spin the sample and take the clear supernatant.

**Assess digestion and ligation efficiency, and DNA concentration 'TIMING' 2-4 h**

**48** The next day, check the digestion and ligation efficiency of the 3C sample. Load 2 µl of the ligated 3C DNA on a 0.8% (w/v) agarose gel), along side with the 30 µl DNA of the digestion efficiency control (see Box 2). Stain the gel with EtBr. To estimate the DNA concentration of the ligated 3C sample, also load a dilution series on gel of genomic DNA with a known concentration and/or a standardized sample (See Fig. 3, Fig. 6 and ANTICIPATED RESULTS).

'CAUTION' EtBr is a mutagen. Wear gloves when handling gels and solutions containing EtBr. Dispose of EtBr gels and solutions according to the regulations

'TROUBLESHOOTING'

**49** The 3C DNA sample is now ready for qPCR analysis [12,15]. We use ~50-100 ng DNA per qPCR reaction.

'PAUSE POINT' In our hands the 3C DNA template can be stored at -20 ºC for 1-2 months.

**'TIMING'**

Steps 1-3, preparation of plant tissue: 15 min

Steps 4-6, formaldehyde crosslinking: 70 min

Steps 7-17, isolation of intact maize nuclei: 1-2 h

Steps 18-25, DNA digestion: 18-20 h

Steps 26-35, Intramolecular ligation: 7-8 h + overnight step

Steps 36-47, DNA purification: 4-5 h + overnight step

Steps 48-49, Assess digestion, ligation, and DNA concentration: 2-4 h

qPCR analysis (not discussed in this Protocol): 2-3 days (depending on the amount of qPCR runs) [12,15]

**'ANTICIPATED RESULTS'**

**Isolation of intact nuclei (Steps 12, 14 and 16)**

3C analysis requires freshly isolated, intact nuclei that are not aggregated. Intact maize nuclei have a spherical shape and the chromocenters and nucleoli are easily visible (left and middle panel in Fig. 2a). The nuclei isolation protocol presented in this paper also yields ample, intact nuclei from *Arabidopsis* leaves (right panel in Fig. 2a), indicating that the protocol is also suitable for other plant tissues.

**Inactivation of endogenous nucleases (step 22)**

When setting up or optimizing the 3C protocol, we recommend checking for the presence of endogenous nucleases. In existing protocols, before addition of a restriction enzyme, nuclei are permeabilized by incubation at 37 ºC with SDS (step 22). Incubation of maize nuclei at these conditions resulted in a nucleosome pattern, in the presence and absence of a restriction enzyme (Fig. 3a, lanes 4 and 5), indicating DNA degradation by endogenous nucleases. The DNA was still intact when isolated from an aliquot taken before the SDS incubation (Fig. 3a, lane 6). Thus, the degradation occurred during the SDS incubation. Nucleases were prevented from degrading the DNA when, after the addition of SDS, the nuclei were first incubated at 65 ºC for 40 min, followed by incubation at 37 ºC (Fig. 3a, lanes 7-9). When incubated at 65 ºC for 60 min, DNA degradation was almost completely absent, but realize that at this temperature also the crosslinks are reversed.

**DNA digestion and ligation efficiency (steps 25, 32 and 48)**

The efficiency of the DNA digestion needs to be as high as possible; preferably more than 80%. Therefore, when setting up 3C, the digestion efficiency should be carefully checked. To this end, take 1/3$^{rd}$ of the sample after overnight digestion in step 25. Reverse the crosslinks and purify the DNA. Separate the DNA on an agarose gel, blot the gel and hybridize the resulting membrane with probes specific for the locus of interest. An example is shown in Fig. 4. In panel b, the digestion efficiencies are shown for conditions described in existing 3C protocols, varying the enzyme (*Bgl*II) concentration. Besides the expected band of ~1.6 kb (indicated with an arrow), numerous larger bands are visible, indicating incomplete digestion. After optimizing the digestion conditions for maize nuclei, the ~1.6 kb band is practically the

only band visible (Fig. 4c). Another method to determine the digestion efficiency uses qPCR and is described by Hagège and colleagues in Box 2 of their paper [12]. Once the optimal digestion conditions have been established, we recommend checking the digestion efficiency by ethidium bromide agarose gel electrophoresis in every 3C experiment (step 32, Box 2). Satellite bands indicate efficient DNA digestion (see e.g. Fig. 3a lane 8, and Fig. 3b D lanes). Compare the digestion pattern to that of a genomic DNA sample (gDNA) digested with the same enzyme (e.g. Fig. 3a lane 2, and Fig. 3c lane 1). Examples of poor digestion are shown in Fig. 3c. In such cases see the TROUBLESHOOTING table.

Like for the digestion efficiency, in every 3C experiment the ligation efficiency must be tested. To this end, run an aliquot taken in step 48 on a 0.8% agarose gel. The aliquot should run as a single band at the top of the gel (Fig. 3b, L lanes). Figure 3d shows an example of poor ligation efficiency. To estimate the DNA concentration, run a dilution series of genomic DNA of a known concentration on the same gel as the digestion and ligation efficiency test samples (Fig. 6).

**Examples of successful and unsuccessful 3C experiments**

3C samples that are well digested and ligated, and contain sufficient DNA are ready to be analyzed by qPCR. Such samples do however not necessarily lead to acceptable qPCR runs. Therefore, after the qPCR data are obtained, the data need to be critically evaluated. An important measure for the quality of a 3C experiment is the detection of a decrease in interaction frequencies when the genomic distance between a sequence fragment of interest and interacting fragments increases, resulting in a curve with a downward slope when the relative crosslinking is plotted against the distance from the element of interest. The interactions between the fragment of interest and neighbouring fragments are due to the occurrence of random collisions, the frequency of which in general decreases with an increasing genomic distance (see ref 23). A practically flat curve therefore indicates an unsuccessful experiment (Figure 7, black curve). The grey curves in Figure 7 do show a relatively high level of interaction frequencies between the fragment of interest (I) and the fragment closest by (IV), and are examples of successful experiments.

Specific physical interactions are defined by a local peak in the curve; a peak in interaction frequencies that is higher than what can be expected based on random collisions. Importantly, a peak in interaction frequencies between a fragment of interest, e.g. the transcription start site, and another fragment can only be reliably detected if neighbouring fragments show significantly lower interaction frequencies with the fragment of interest (Fig.

7, grey curves, e.g. compare the interaction frequencies with fragment XI, X and IX). Therefore, when setting up the qPCR to analyze the 3C material, it is important to realize that a sufficient number of data points needs to be examined to reliably detect chromosomal interactions. Once physical interactions are observed for a fragment of interest, the best way to verify these interactions is by performing reciprocal experiments using the interacting fragments as a fragment of interest [1,14].

'TROUBLESHOOTING'

Troubleshooting advice can be found in Table 1.

**'ACKNOWLEDGEMENTS'**

The authors would like to thank Robert-Jan Palstra and Petra Klous for practical assistance in setting up 3C in plants. Laurens Bossen is thanked for providing the *Arabidopsis* picture. M. Stam was supported by the Royal Netherlands Academy of Arts and Sciences (KNAW).

**'COMPETING INTERESTS STATEMENT'** The authors declare they have no competing financial interests.

**'REFERENCES'**

1. Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F. & deLaat, W. Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* **10**, 1453-1465 (2002).
2. Horike, S., Cai, S., Miyano, M., Cheng, J.F. & Kohwi-Shigematsu, T. Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat Genet* **37**, 31-40 (2005).
3. Bacher, C.P. et al. Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation. *Nat Cell Biol* **8**, 293-9 (2006).
4. Xu, N., Tsai, C.L. & Lee, J.T. Transient homologous chromosome pairing marks the onset of X inactivation. *Science* **311**, 1149-52 (2006).
5. Lanzuolo, C., Roure, V., Dekker, J., Bantignies, F. & Orlando, V. Polycomb response elements mediate the formation of chromosome higher-order structures in the bithorax complex. *Nat Cell Biol* **9**, 1167-74 (2007).
6. Fransz, P.F. et al. Integrated cytogenetic map of chromosome arm 4S of A. thaliana: structural organization of heterochromatic knob and centromere region. *Cell* **100**, 367-76 (2000).
7. Carter, D., Chakalova, L., Osborne, C.S., Dai, Y.F. & Fraser, P. Long-range chromatin regulatory interactions *in vivo*. *Nat Genet* **32**, 623-6 (2002).
8. Dekker, J., Rippe, K., Dekker, M. & Kleckner, N. Capturing chromosome conformation. *Science* **295**, 1306-11 (2002).
9. Pecinka, A. et al. Tandem repetitive transgenes and fluorescent chromatin tags alter local interphase chromosome arrangement in *Arabidopsis thaliana*. *J Cell Sci* **118**, 3751-8 (2005).
10. Nagano, T. et al. The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. *Science* **322**, 1717-20 (2008).
11. Murakami, K., Oshimura, M. & Kugoh, H. Suggestive evidence for chromosomal localization of non-coding RNA from imprinted LIT1. *J Hum Genet* **52**, 926-33 (2007).

12. Hagege, H. et al. Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat Protoc* **2**, 1722-33 (2007).
13. Splinter, E., Grosveld, F. & de Laat, W. 3C technology: analyzing the spatial organization of genomic loci in vivo. *Methods Enzymol* **375**, 493-507 (2004).
14. Louwers, M. et al. Tissue- and Expression Level-Specific Chromatin Looping at Maize b1 Epialleles. *Plant Cell* (2009).
15. Splinter, E. et al. CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. *Gene Develop* **20**, 2349-2354 (2006).
16. Simonis, M., Kooren, J. & de Laat, W. An evaluation of 3C-based methods to capture DNA interactions. *Nat Methods* **4**, 895-901 (2007).
17. Simonis, M. et al. Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nat Genet* **38**, 1348-54 (2006).
18. Zhao, Z. et al. Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet* **38**, 1341-7 (2006).
19. Dostie, J. et al. Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. *Genome Res* **16**, 1299-309 (2006).
20. Cai, S., Lee, C.C. & Kohwi-Shigematsu, T. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat Genet* **38**, 1278-88 (2006).
21. Fullwood, M.J. & Ruan, Y. ChIP-based methods for the identification of long-range chromatin interactions. *J Cell Biochem* **107**, 30-9 (2009).
22. Fullwood, M.J., Wei, C.L., Liu, E.T. & Ruan, Y. Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. *Genome Res* **19**, 521-32 (2009).
23. Dekker, J. The three 'C' s of chromosome conformation capture: controls, controls, controls. *Nat Methods* **3**, 17-21 (2006).

**'LEGENDS'**

**Figure 1.** Schematic diagram, outline and timing of the 3C-qPCR procedure.

The chromatin conformation within the nuclei is fixed by formaldehyde, followed by restriction digestion and intramolecular ligation. The ligation products are purified and quantified using qPCR. The qPCR part of the procedure is not discussed in this Protocol. **(a)** A chromosomal locus of interest is indicated in grey. A regulatory element is indicated in green and a promoter region in magenta. The arrow indicates the transcription start. **(b)** The outline and timing of the 3C-qPCR procedure. The asterisks indicate the steps at which aliquots are taken to test the nuclei quality and quantity (1), restriction digestion efficiency (2), ligation efficiency and DNA concentration (3).

**Figure 2.** Plant nuclei before and after SDS treatment.

Image acquisition was carried out on a Leitz Aristoplan (Leica Microsystems AG, Wetzlar, Germany) microscope with filters for DAPI. Images were captured with a CCD camera (Apogee, Logan, UT) using ImageProPlus Software (Media Cybernetics, Silver Spring, MD). The images were digitally processed using Adobe Photoshop (Adobe, San Jose, CA).

(**a**) Typical example of intact plant nuclei stained with DAPI (see Box 1). Chromocenters (DAPI-dense) and nucleoli (dark regions) are visible. The left and middle panels each display a maize nucleus; on the right is an example of a nucleus derived from an *Arabidopsis* leaf using the nuclei isolation protocol described in this paper.

(**b**) Typical example of maize nuclei stained with DAPI after SDS treatment (see Box 1). Nuclei are permeabilized by the SDS treatment. Their round shape is lost, giving rise to distorted, partially disintegrated nuclei (left panel) that often tend to aggregate (right panel).

**Figure 3.** Digestion and ligation efficiency test samples run on a 0.8% agarose gel and stained with EtBr.

Keys: M = marker; gDNA = genomic DNA; N = nuclei; *B* = *Bgl*II enzyme; C = control sample showing sufficient ligation; D = digestion efficiency test sample; L = ligation efficiency test sample. In each panel, the numbers following a D or L refer to a particular sample in a given 3C experiment.

(**a**) DNA degradation during SDS incubation at 37 ºC. Lane 1 contains a DNA size marker. Lane 2 contains genomic maize DNA digested with *Bgl*II, a positive control for restriction digestion: satellite bands are clearly visible, indicating complete digestion. Lane 3 contains undigested gDNA, the negative control for digestion. Lane 4 and 5 show that during SDS

treatment, the DNA degrades. Nuclei were treated with SDS for 60 min at 37 ºC, followed by Triton X-100 treatment and overnight (O/N) incubation with *Bgl*II (lane 4) or no enzyme (lane 5). The next day, the crosslinks were reversed and the DNA purified. In both lanes a clear nucleosome pattern is visible, even in the absence of *Bgl*II, indicating that the migration pattern in both lanes is due to DNA degradation. Lane 6 shows that the DNA is not degraded prior to SDS incubation. Nuclei were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 5 mM MgCl2; 0.1 mM EGTA; 1x complete protease inhibitor) and the crosslinks reversed O/N. Lane 7 shows that nucleases can be inactivated to a large extent. Nuclei were treated with SDS for 30 min at 65 ºC, followed by 30 min at 37 ºC, treatment with Triton X-100 and O/N incubation at 37 ºC. The following day, the crosslinks were reversed and the DNA purified. Compared to lane 5, most of the DNA is at the top of the gel, indicating it is intact. Nuclei in lane 8 received the same treatment as those in lane 7, except that *Bgl*II was added prior to O/N incubation at 37 ºC. The DNA is well digested, satellite bands are visible. Lane 9 shows DNA isolated from nuclei treated with SDS for 60 min at 65 ºC, followed by Triton X-100 treatment and O/N incubation at 37 ºC. Even less DNA decay is visible than in lane 7. 65 ºC is however also the temperature at which crosslinks are reversed. To prevent both DNA decay and the reversal of crosslinks, in the protocol presented, nuclei are treated with SDS for only 40 min at 65 ºC, then for 20 min at 37 ºC (see step 22 in PROCEDURE), followed by Triton-X treatment and restriction digestion.

(**b**) Panel b shows a gel with four 3C samples that were all efficiently digested with *Bgl*II (Lanes 2, 4, 6 and 8; satellite bands visible, compare with panel a, lane 2) and subsequently ligated (Lanes 3, 5, 7 and 9). Lane 1 contains a positive control sample for the ligation. This sample was identified as being well ligated (DNA runs as one band at the top of the gel) and loaded on every gel for comparison.

(**c**) Panel c shows an example of poor digestion efficiency. Lanes 1 and 2 contain the positive and negative control for digestion, respectively. Lane 3 contains the positive control for the ligation. Although samples 1 and 2 seem well ligated (lanes 5 and 7, respectively), the digestion test samples (lanes 4 and 6, respectively) show they were poorly digested, indicating that these samples could not be used for 3C analysis.

(**d**) Panel d shows an example of poor ligation efficiency. Lane 1 indicates efficient restriction digestion, while lane 2 shows that the sample was practically not ligated. Such a sample cannot be used for 3C analysis.

**Figure 4.** Optimization of the restriction digestion measured by DNA blot analysis.

(**a**) Restriction map of part of the maize *b1* locus. Expected *Bgl*II fragment sizes are indicated. The black bar represents the probe used in the DNA blot analysis. The arrow shows the transcription start site. B = *Bgl*II restriction site.

(**b**) Restriction digestion efficiency before (plant-specific) optimization of the protocol. Nuclei were digested with varying amounts of *Bgl*II restriction enzyme. In case of complete digestion, hybridization of the probe to the DNA blot would result in a single 1.6 kb band, the size of which is indicated with an arrow. Although such a band is present in most lanes, several larger bands resulting from partial digestion are present as well.

(**c**) Restriction digestion efficiency using the protocol presented in this paper. This DNA blot depicts a similar experiment as described in panel b, except that in this experiment in all lanes only the expected 1.6 kb band (arrow) is present, indicating high digestion efficiency. Keys: U = units of *Bgl*II restriction enzyme; M = marker

**Figure 5.** A schematic representation of the maize endogenous control locus, *Sam.* The transcription start site is indicated with a hooked arrow (0 kb). *Bgl*II sites are indicated by *B.* The two triangles indicate the location of the primers used for the 3C-qPCR analyses.

**Figure 6**. Determination of the DNA concentration of a 3C sample.

To determine the concentration of a 3C sample, an aliquot of a 3C sample is run on a 0.8% agarose gel along side a dilution series of undigested genomic DNA (gDNA) of known concentration (280 ng $\mu l^{-1}$). The gel is stained with EtBr and visualized under UV light. The ligated 3C sample and the undigested genomic reference sample run as a single, high-molecular-weight band at the top of the gel.

Lane 1, 30 µl digested 3C sample from step 32 (Box 2); Lane 2, 2 µl ligated 3C sample from step 48; Lane 3: 4 µl gDNA; Lane 4, 2 µl gDNA; Lane 5, 1 µl gDNA; Lane 6, 0.5 µl gDNA; Lane 7, 0.25 µl gDNA; Lane 8: 0.125 µl gDNA.

**Figure 7.** Examples of successful and unsuccessful 3C-qPCR analyses of long-distance interactions at the maize *b1* locus.

The normalized level of each ligation product (*Bgl*II fragments IV to XII) has been plotted against the distance (in kb) from the *b1* transcription start site (TSS; see map below graph). For details on the experimental setup, PCR primers, TaqMan probe, and normalization, see Louwers et al. (2009). The locus-wide crosslinking frequencies were determined for the

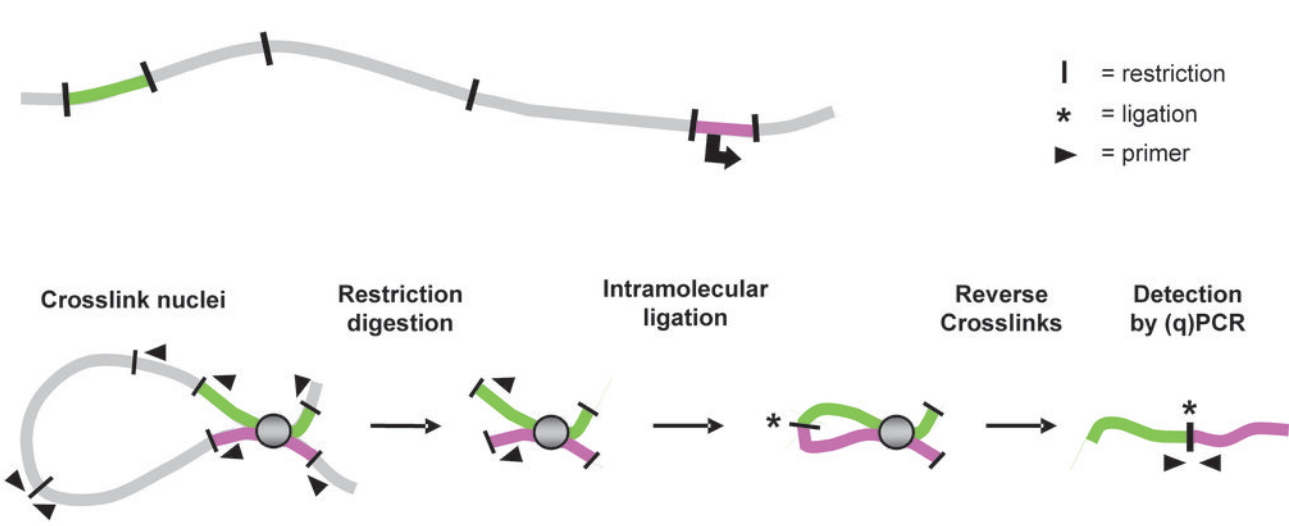
fragment containing the TSS (I); this fragment of interest has been indicated by a black vertical bar. The arrowheads on the map indicate a known enhancer region. Three independent 3C-qPCR experiments were performed on three different, but genotypically identical, maize husks. The DNA was cut with *Bgl*II. All three samples showed satisfactory digestion and ligation efficiencies and yielded enough DNA for qPCR analysis. Sample 1 (black curve) resulted in a basically flat curve, while samples 2 and 3 (grey curves) resulted in very similar curves showing various peaks in interaction frequencies, one of which (fragment X) involving the enhancer region. The curves of sample 2 and 3, but not that of sample 1, show the predicted relatively high level of interaction frequencies between the fragment of interest (I) and the fragment closest by (IV). Besides that, the curve of sample 1 is very different from that of sample 2 and 3, and other samples analyzed (data not shown). Together, these data indicate that sample 2 and 3 exemplify a successful 3C experiment, and sample 1 an unsuccessful experiment. A step different from digestion and ligation, e.g. the crosslinking, must have gone wrong.

**Table 1.** Troubleshooting table.

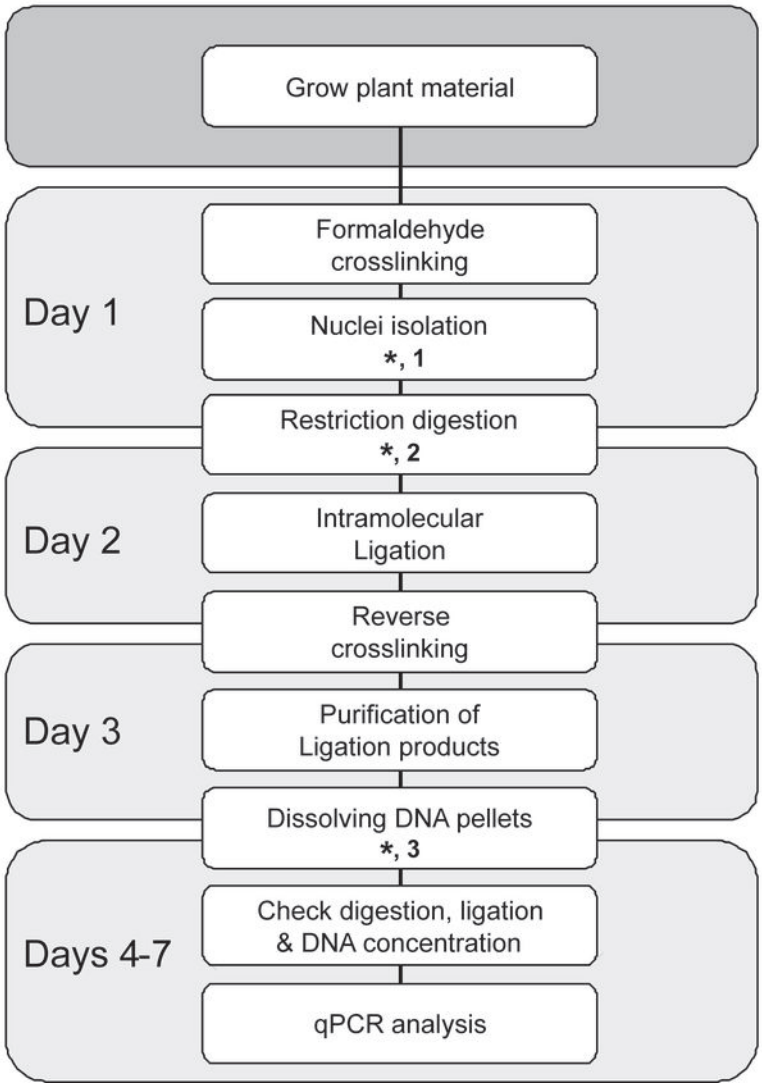
| step | problem | possible reason | solution |
|---|---|---|---|
| 5 | Insufficient fixation | Buffer hampers fixation | The buffer used to isolate and fix the nuclei should not contain Tris-HCl, even though many published nuclei isolation/fixation buffers do. Tris contains reactive amines that crosslink formaldehyde to Tris, leaving less formaldehyde to fix DNA and proteins. Furthermore, the presence of EDTA and EGTA in the isolation buffer is not compatible with the presence of $Mg^{2+}$ ions: $Mg^{2+}$ is required to stabilize DNA in chromatin |
| | | Fixation time is too short, or the concentration of the crosslinking agent is too low | To optimize the fixation conditions, perform a time and dilution series and check if the ligation is saturated by (q)PCR [13] |
| | Insufficient fixation: tissue is not translucent after fixation | Too much tissue in the tube | Use less tissue per sample. Pool samples after the fixation step if necessary |
| | Too much fixation | Fixation time is too long, or the concentration of the crosslinking agent is too high | To optimize the fixation conditions, perform a time and dilution series and check if the ligation is saturated by (q)PCR [13] |
| 9 | Difficulties to grind the tissue to a fine powder | Tissue is too wet | Grinding wet plant tissue with liquid nitrogen is difficult and not efficient. The tissue needs to be quite dry to enable efficient grinding. We advise to pat the tissue dry between paper towels |
| 12 and Box 1 | Lots of debris surrounding the nuclei | Poor filtration of the nuclei suspension | Never squeeze the filter to speed up the filtration process. This will result in contamination of the nuclei preparation with excess debris of for example pieces of cell wall and cytoplasm |
| 14 | Nuclei pellet does not dissolve well | | Use a cut-off P1000 pipette tip and gently pipette the nuclei up and down. Alternatively, a Potter tube can be used to dissolve big clumps of nuclei. Usually, these methods do not negatively affect the quality of the nuclei |
| | Pellets of different samples have various colors | Coloring is due to the amount of starch and anthocyanins in the tissue used | Pelleted maize nuclei can have different colors: usually they are green, but sometimes they are white or purple. White indicates starch, purple indicates the presence of anthocyanins. The exact color depends on the pH value of the solution. The different colors do not seem to affect the quality of the nuclei or 3C efficiency. Do not try to wash away the color; this will only result in a lower yield of nuclei |
| 14 and Box 1 | Broken, sheared nuclei | Centrifugation speed is too high | Do not spin the nuclei in a Sorvall Centrifuge at a speed higher than 4000-5000*g* (Both 8000*g* and 12000*g* were tested and resulted in broken, sheared nuclei) |
| | Low yield of nuclei | Too many washing steps | Limit the number of washing steps. More than two washing steps easily results in a low yield, and a high proportion of damaged nuclei |

| | | | |
|---|---|---|---|
| 25 | No or partial digestion (DNA blot analysis; Fig. 4b) | Nuclei aggregate during the digestion | Increase the digestion volume up to 2 ml, instead of performing the digestion in 0.5 ml |
| | | The digestion efficiency of particular enzymes is hampered by presence of SDS | *Bam*HI, e.g., is very sensitive to SDS. The conditions described in this protocol work well for *Bgl*II, *Hind*III and *EcoR*I. For *Bam*HI, the final SDS concentration to permeabilize the nuclei should be lowered to 0.15% (instead of 0.3%) |
| | | Incubation time for restiction digestion is too short | It is important to allow sufficient time for digestion (16 h at least) |
| | | Addition of spermidine | The use of spermidine (final: 8 mM) is known to stimulate restriction digestions. With our 3C experiments, the addition of spermidine however negatively affected the digestion; the DNA was not digested at all |
| 25, 32 and Box 2 | No or incomplete digestion (DNA blot and/or EtBr-gel analysis; Figs. 3c and 4b) | Triton X-100 solution too old or decayed by light | Triton X-100 is light-sensitive. Therefore, keep your solution in the dark. In addition, frequently prepare a new work solution. An old solution has a notable negative effect on the digestion efficiency |
| 32 and Box 2 | Digestion efficiency aliquot (step 32) does not yield enough DNA for gel analysis | | To check the digestion efficiency, a 30 µl aliquot can also be taken directly after step 25, followed by reversal of the crosslinks overnight. In our hands, however, the DNA precipitated more efficiently when taking a 300 µl aliquot out of the 7.5 ml sample at step 32 |
| 37 | Insufficient separation of Phenol-Chloroform and aqueous phases | Phenol-Chloroform-Isoamylalcohol is too cold | Make sure the Phenol-Chloroform-Isoamylalcohol (25:24:1) is at room temperature. The separation of nucleic acids from proteins is better at room temperature; 4 °C increases the possibility of a phase inversion. We recommend placing the Phenol-Chloroform-Isoamylalcohol solution at room temperature the night before |
| | Difficult to pipette off the aqueous phase | Interphase is not well defined | Eppendorf's Phase-Lock Gel system for phenol extractions can be used for an easier separation (Eppendorf, PLG, Light, 50 ml, 0032.005.306) |
| 43 | No or insufficient DNA precipitation | | Add a DNA carrier, such as glycogen or switch the brand of polypropylene tubes used |
| 48 | Ligation efficiency is insufficient (Fig. 3d) | SDS concentration is too high to allow efficient ligation | SDS reduces the ligation efficiency dramatically. If the ligation does not work well, lower the final SDS concentration used to inactivate the restriction enzyme to 0.35%. Under these conditions the restriction enzyme can still be inactivated and the ligation may work better. We, however, recommend not to lower the SDS concentration unnecessarily to avoid an inefficient inactivation of the restriction enzyme |
| | | SDS is not adequately sequestered by Triton X-100 | The quality of the Triton X-100 solution hugely affects the ligation efficiency. It is crucial to use a relatively freshly made Triton X-100 work solution to quench all the SDS (see also troubleshooting for steps 25 and 32) |
| | | Ligation time was not sufficient | Allow 5 h or more ligation time at 16 °C |

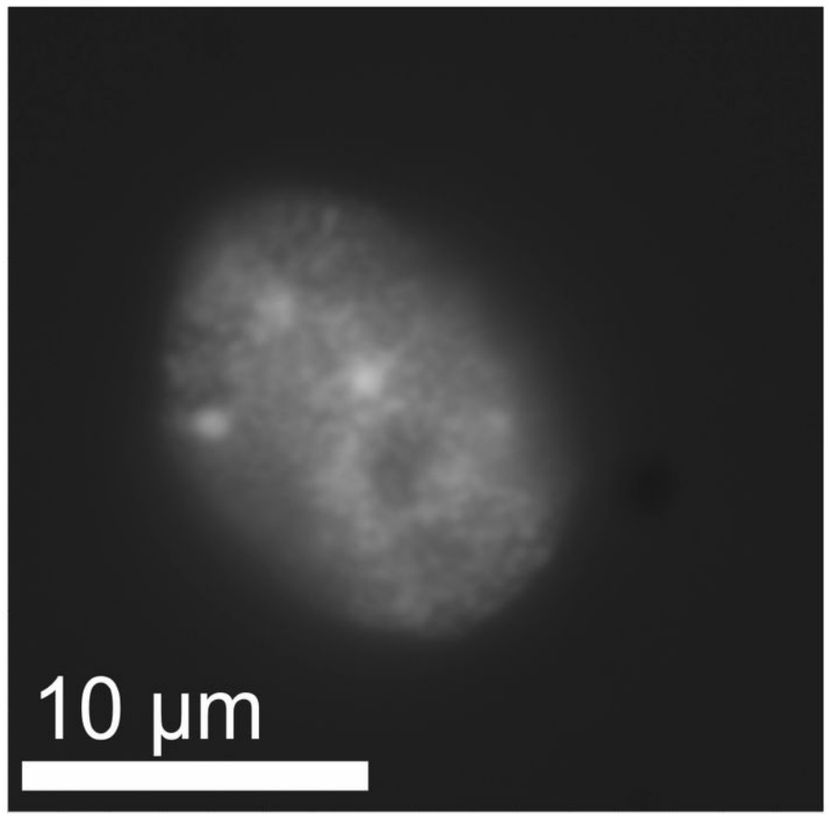
a

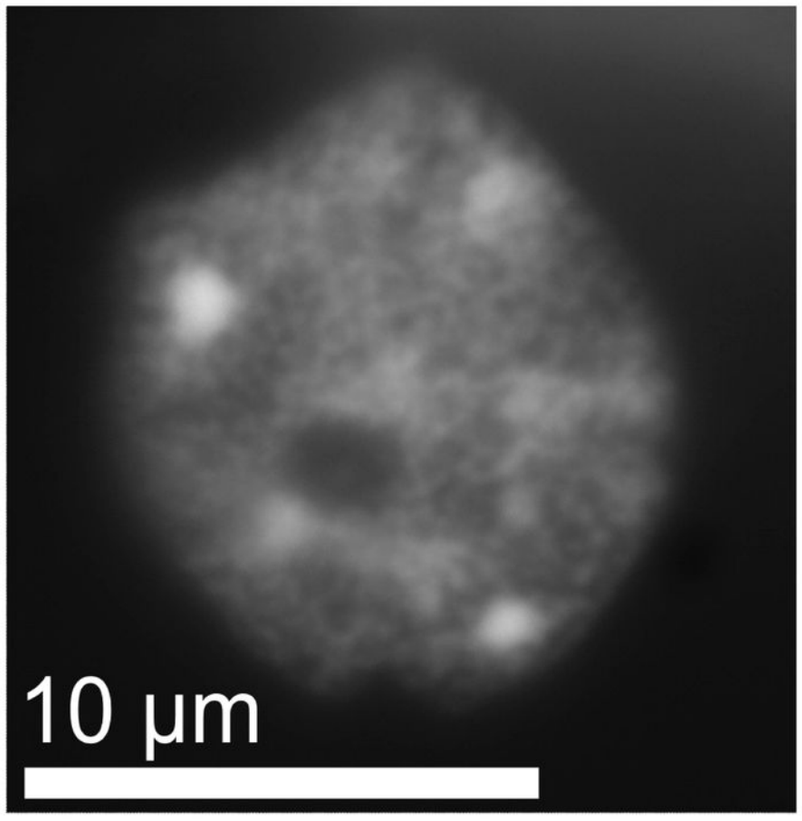

= restriction
= ligation
= primer
Crosslink nuclei
Restriction digestion
Intramolecular ligation
Reverse Crosslinks
Detection by (q)PCR

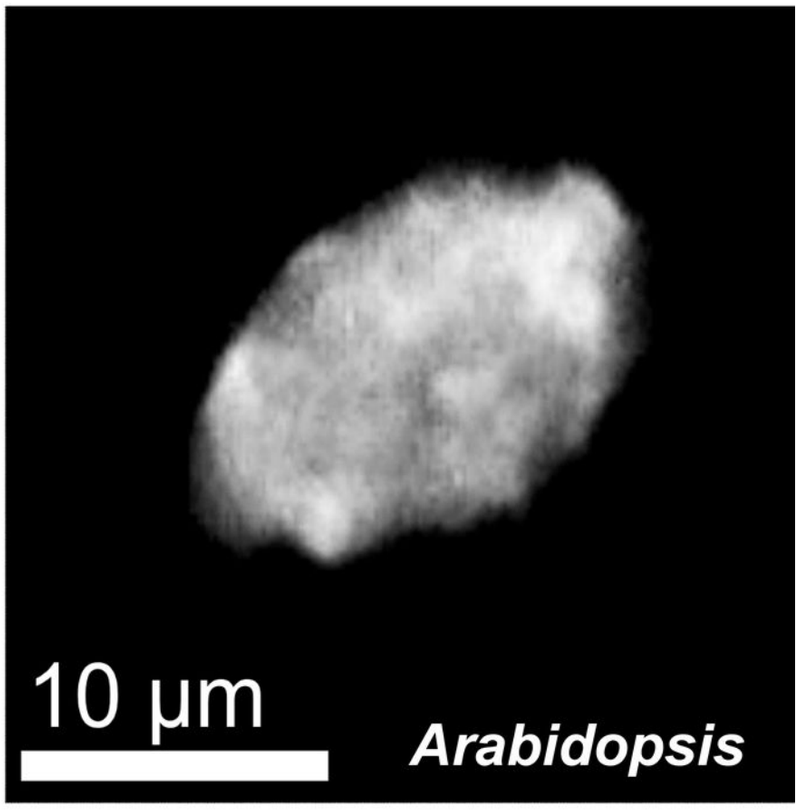
b


Grow plant material
Day 1
Formaldehyde crosslinking
Nuclei isolation
*, 1
Restriction digestion
*, 2
Day 2
Intramolecular Ligation
Reverse crosslinking
Day 3
Purification of Ligation products
Dissolving DNA pellets
*, 3
Days 4-7
Check digestion, ligation & DNA concentration
qPCR analysis

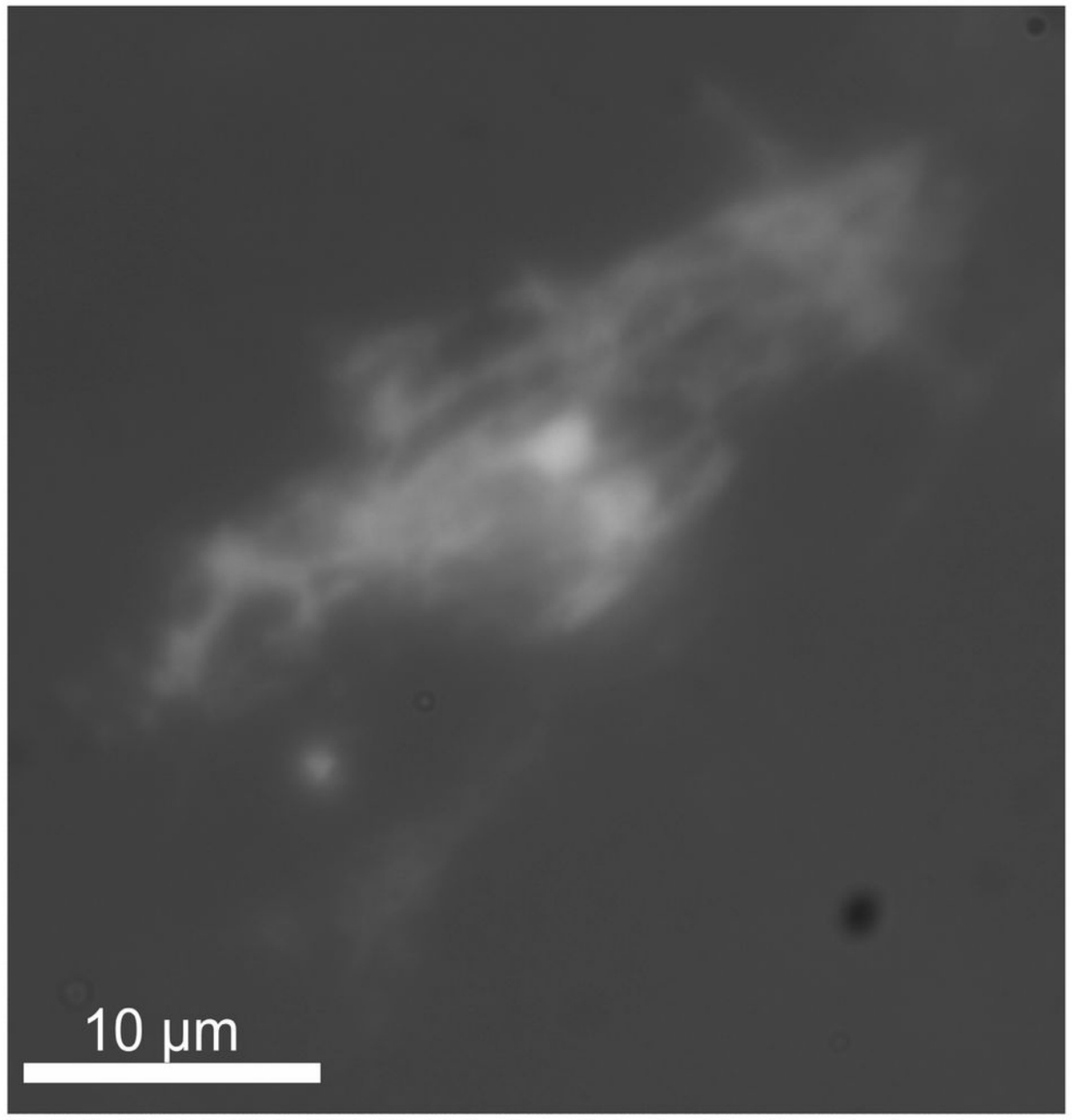
a

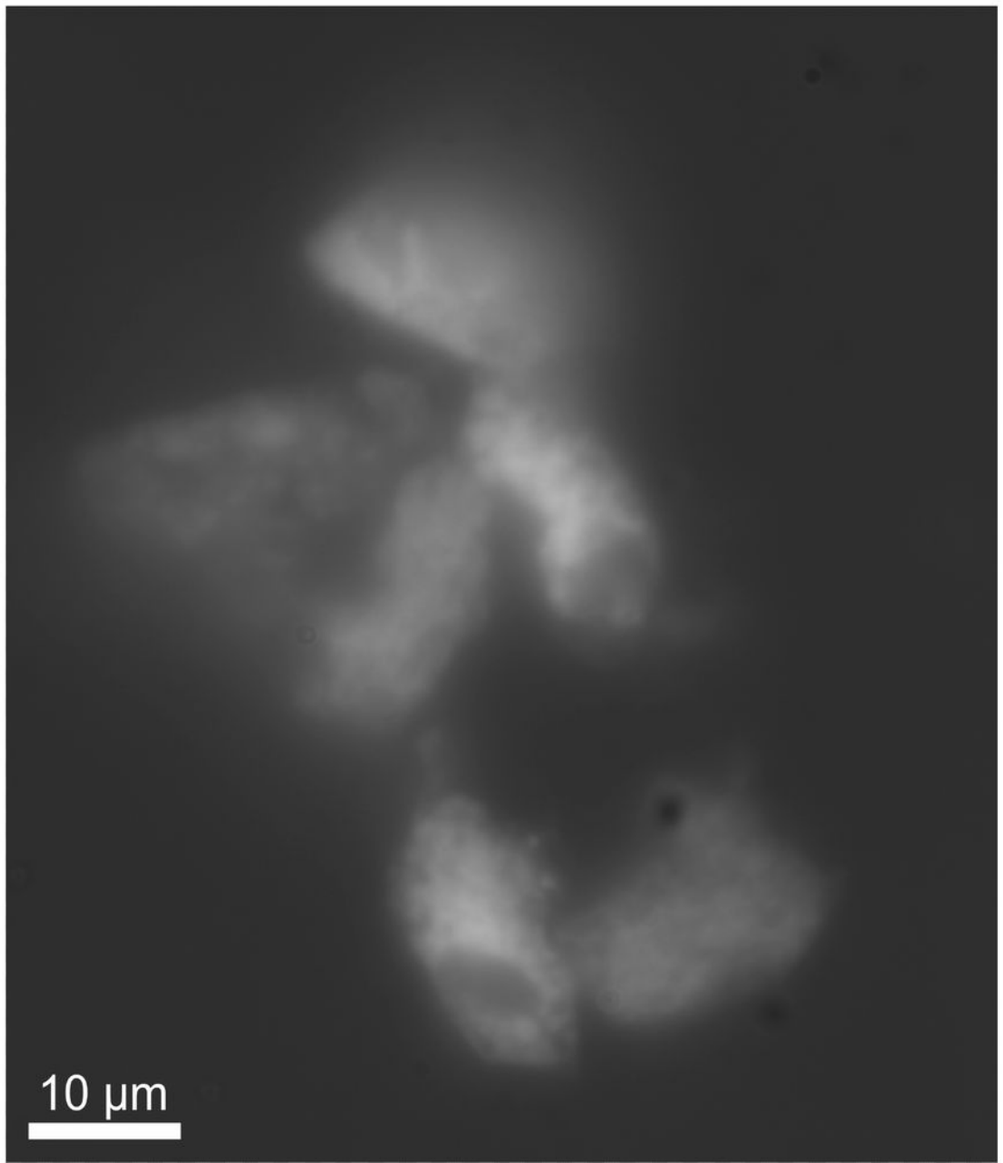


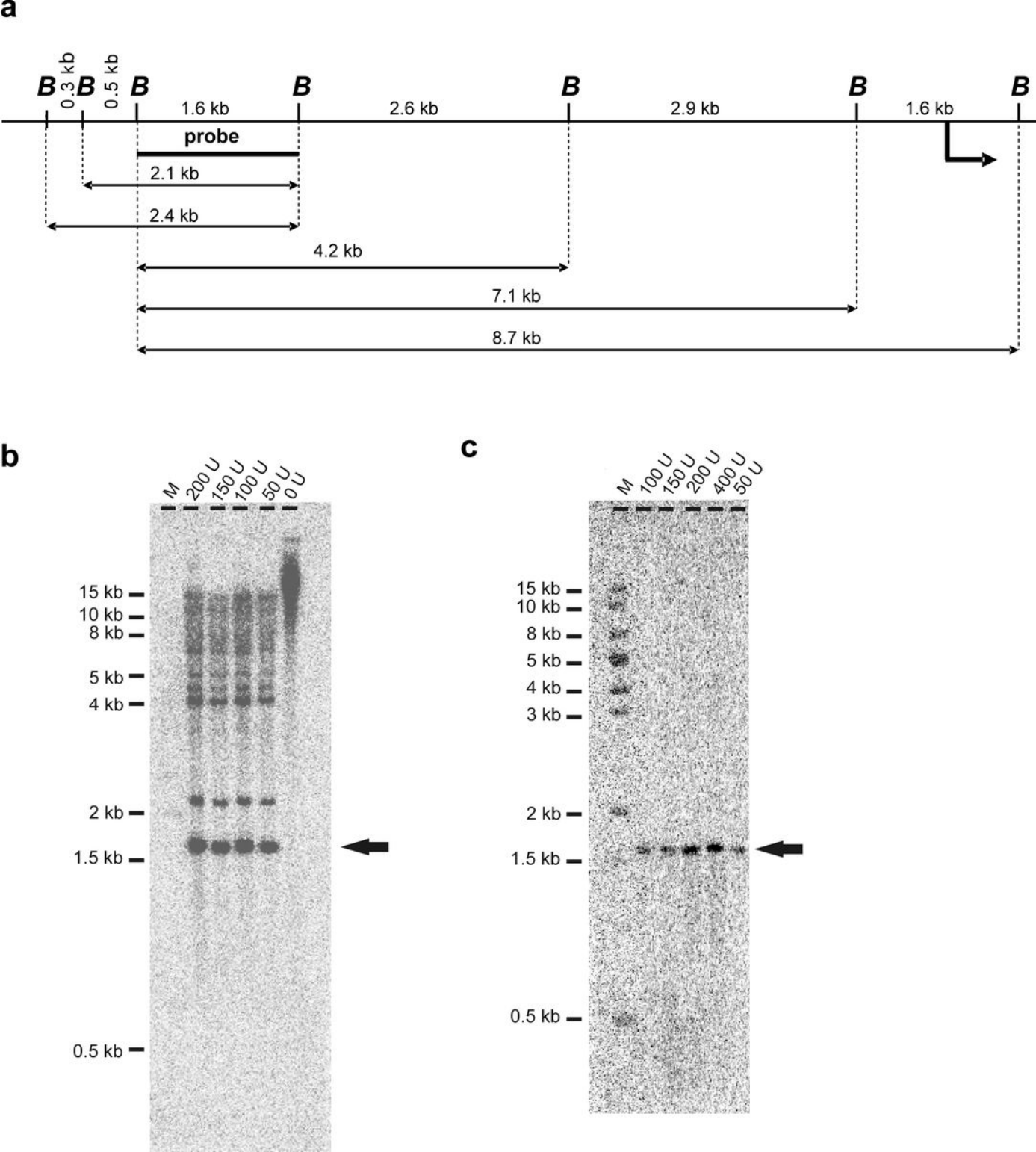



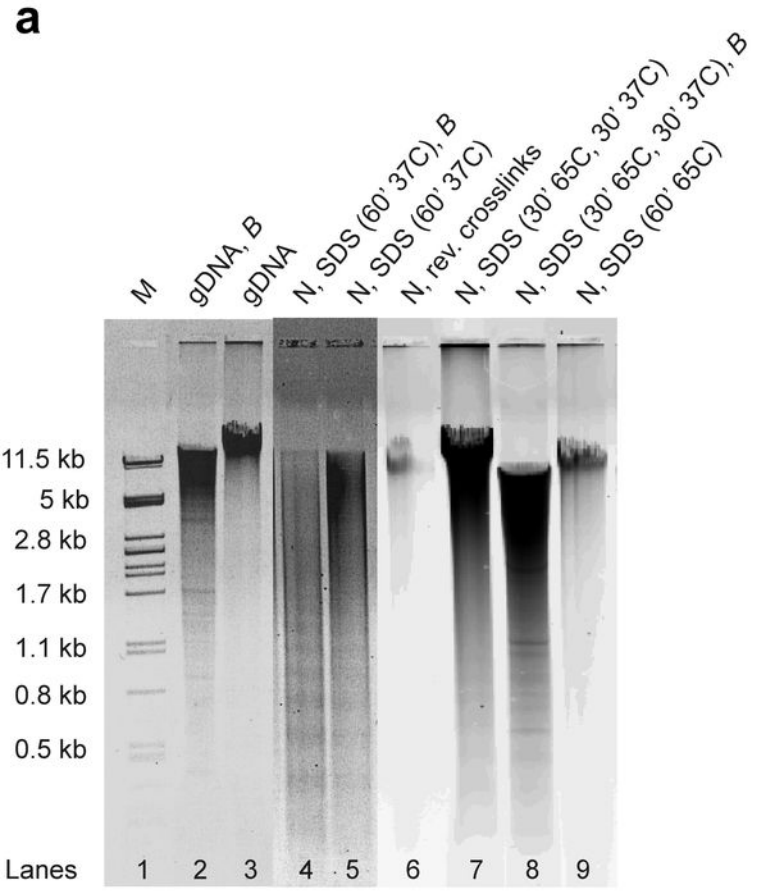



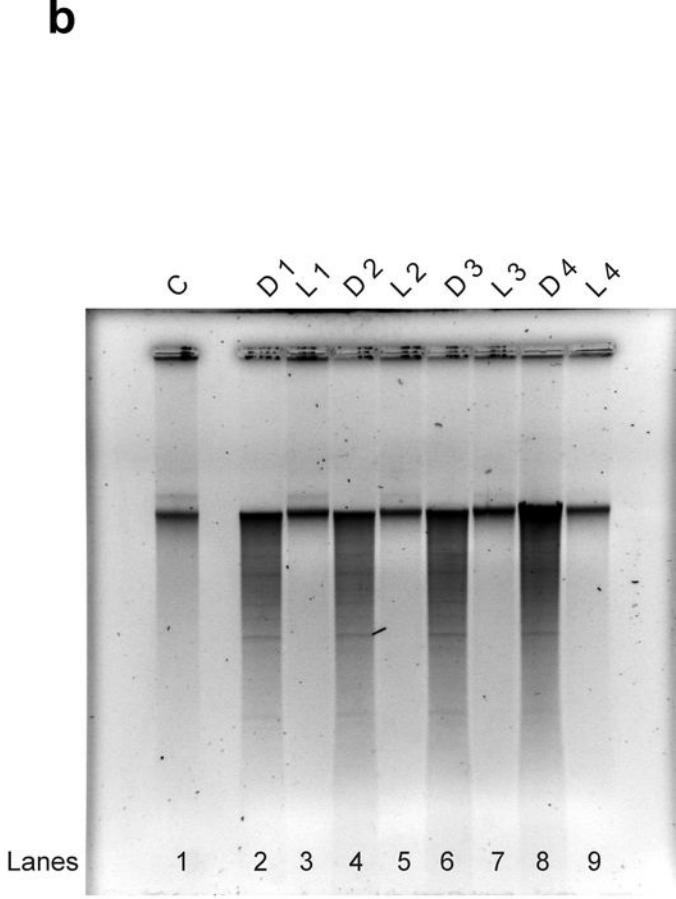

b

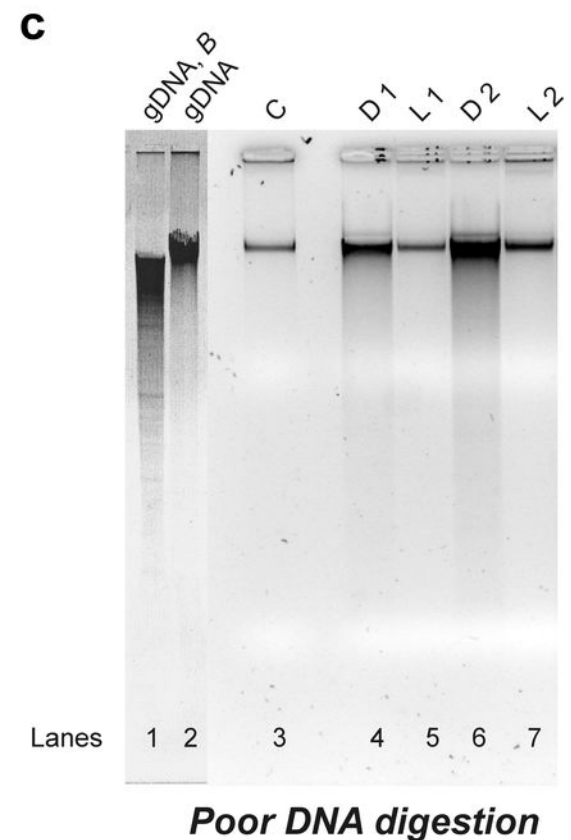


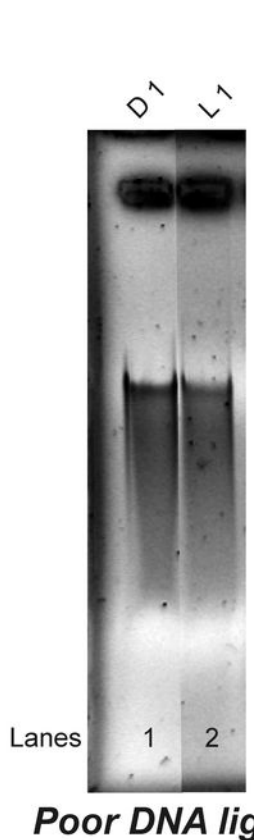



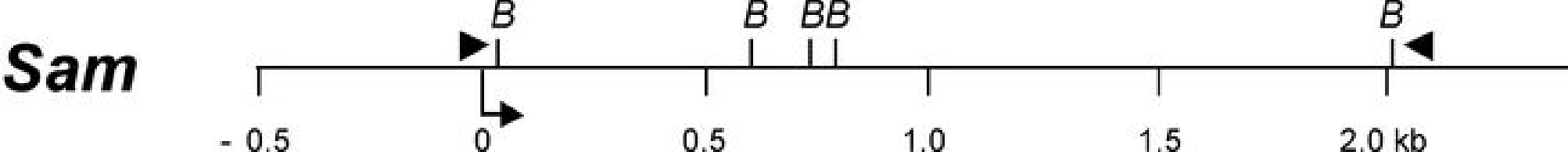

a
B
0.3 kb
B
0.5 kb
B
1.6 kb
B
2.6 kb
B
2.9 kb
B
1.6 kb
B
probe
2.1 kb
2.4 kb
4.2 kb
7.1 kb
8.7 kb
b
M
200 U
150 U
100 U
50 U
0 U
15 kb
10 kb
8 kb
5 kb
4 kb
2 kb
1.5 kb
0.5 kb
c
M
100 U
150 U
200 U
400 U
50 U
15 kb
10 kb
8 kb
5 kb
4 kb
3 kb
2 kb
1.5 kb
0.5 kb

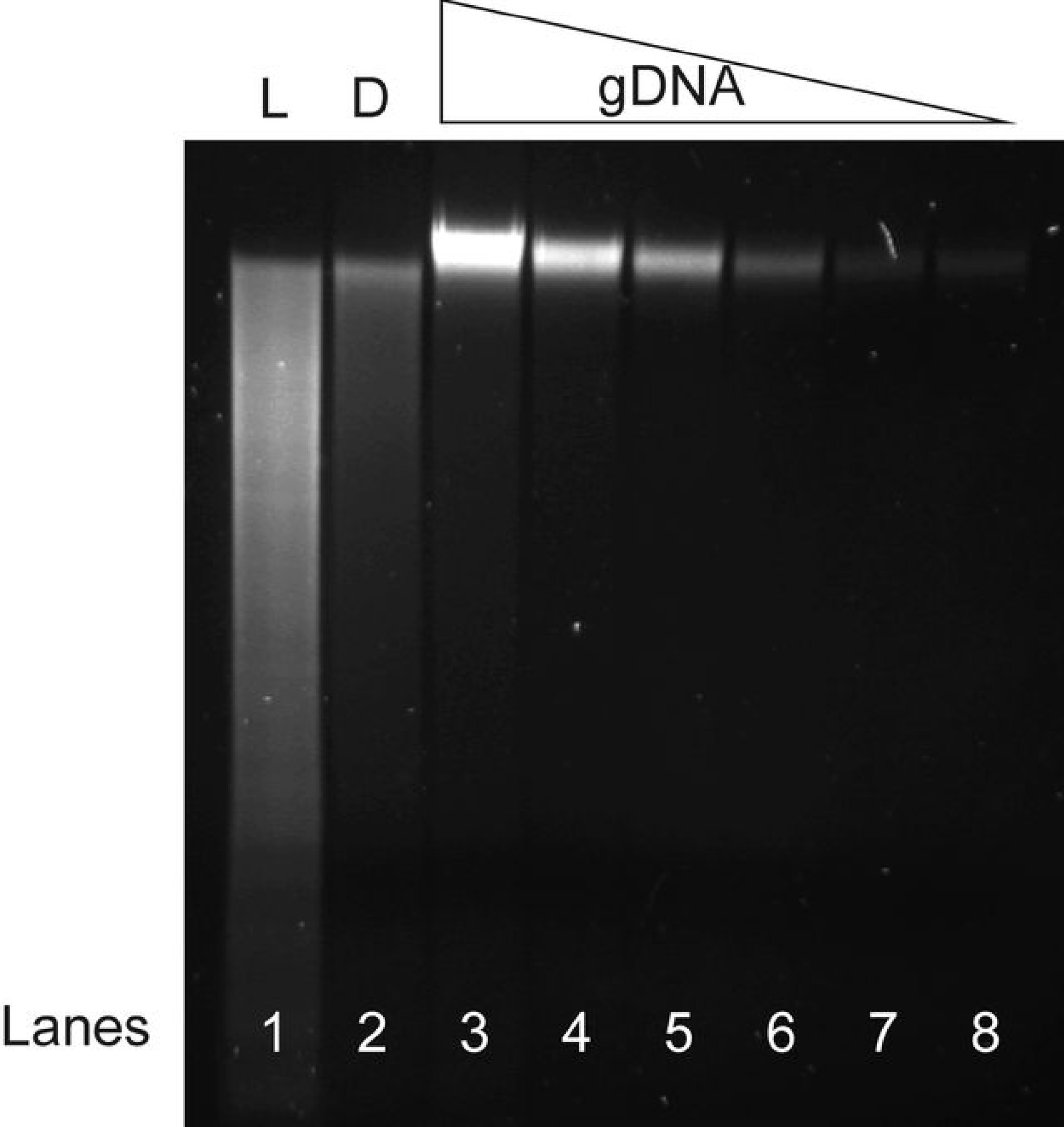

a
M
gDNA, B
gDNA
N, SDS (60' 37C), B
N, SDS (60' 37C)
N, rev. crosslinks
N, SDS (30' 65C, 30' 37C)
N, SDS (30' 65C, 30' 37C), B
N, SDS (60' 65C)
11.5 kb
5 kb
2.8 kb
1.7 kb
1.1 kb
0.8 kb
0.5 kb
Lanes 1 2 3 4 5 6 7 8 9
DNA degradation

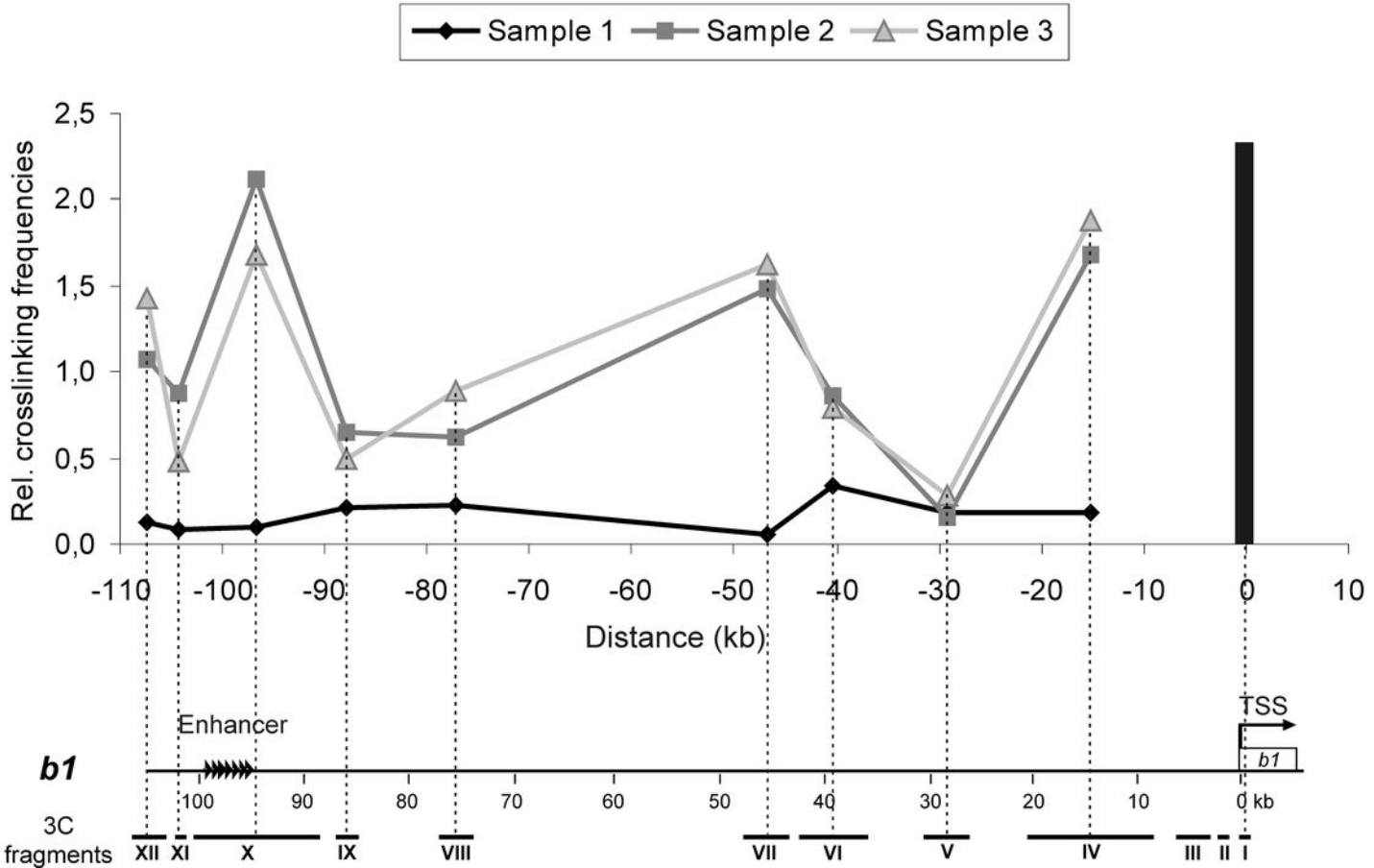

b
C
D 1
L 1
D 2
L 2
D 3
L 3
D 4
L 4
Lanes 1 2 3 4 5 6 7 8 9
Good DNA digestion & ligation


c
gDNA, B
gDNA
C
D 1
L 1
D 2
L 2
Lanes 1 2 3 4 5 6 7
Poor DNA digestion


d
D 1
L 1
Lanes 1 2
Poor DNA ligation


Sam
B
B
B
B
- 0.5
0
0.5
1.0
1.5
2.0 kb


L
D
gDNA
Lanes
1
2
3
4
5
6
7
8


Sample 1
Sample 2
Sample 3
Rel. crosslinking frequencies
2,5
2,0
1,5
1,0
0,5
0,0
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0
10
Distance (kb)
Enhancer
TSS
b1
b1
100
90
80
70
60
50
40
30
20
10
0 kb
3C fragments
XII
XI
X
IX
VIII
VII
VI
V
IV
III
II
I