DSB resection assay **(As described in Zierhut and Diffley, EMBO J 27, 1875-1885, 2008)**

DNA extraction and BstUI digestion

DNA extractions

$2x10^7$ cells were harvested and frozen on dry ice. Cells were stored at –80°C until use. For DNA preparations, cells were thawed on ice and resuspended in 500µl extraction solution (1% SDS; 100mM NaCl; 10mM EDTA; 50mM Tris-Cl pH 8.0; 1% v/v β-mercaptoethanol; 1u/µl lyticase) and lysed by incubation with shaking at 37°C for 6min. Cells were then extracted twice with equal volumes of phenol/chloroform/iso-amylalcohol (25:24:1) and once with chloroform. Phenol saturated with Tris base was used (Rathburn) and phase lock gel tubes (Eppendorf) were used for extractions. Two volumes of 99% ethanol were added to the last aqueous phase, and nucleic acids were precipitated by incubation at –20°C for ~30min. Tubes were then centrifuged (13krpm ~8sec) and the supernatant was discarded. Care was taken to take out as much of the liquid as possible. Pellets were dried for 1min at 37°C and then resuspended in TE pH 8.0 containing 0.05µg/µl RNase A (40µl for samples from G1-arrested cells, 70µl for samples from M-arrested cells). NOTE: Only spin, dry and resuspend a max of 2 samples at a time, otherwise the danger of overdrying is too high! Store resuspended samples on ice until all samples are ready. RNA was digested by incubation for 45min at 37°C with shaking. DNA extraction and RNase digestion were confirmed by running 1µl on an agarose gel. Samples were then stored at –20°C until use.

BstUI digestion

4µl of the DNA extracts were digested in a total volume of 30µl containing 10u of BstUI in 1xbuffer 2 (both New Englands Biolabs). Reactions were allowed to proceed for 1hr at 60°C in a PTC-200 temperature cycler (MJ Research) with lid heating. In mock digests, BstUI was replaced with water. Samples were cooled on ice, centrifuged and then serially diluted three times in 1xTE pH 8.0, first 1:4 and then twice 1:2. The diluted samples, which were to be used as template in qPCR reactions, were stored at –20°C. Filter tips were used throughout, and samples were always kept on ice during the dilutions.

PCR analysis

qPCR was performed with the ABI7000 Sequence Detection System and corresponding software (Applied Biosystems). 1x ABsolute QPCR ROX Mix (ABgene) was used for the reactions. Table 1 lists the oligonucleotides for each amplicon and their respective concentrations within the PCR reaction mix. All labelled oligonucleotides (the TaqMan^{R} probes) were obtained from ABI. All other oligonucleotides were obtained from Sigma Genosys. Reaction volumes were 40µl, containing 4µl of the diluted samples each. The temperature cycling program was 95°C 15min → 45 x (95°C 15sec → 60°C 1min). Primer and probe concentrations were optimised as instructed by ABI qPCR manuals.

For PCR analysis, the base line was set to cycles 6-15, and the following fluorescence values determined the threshold cycle (see below):

Amplicon 0.3kb:	0.16
Amplicon 9kb:	0.2
Amplicon 14kb:	0.2
Control amplicon:	0.1

Analysis of break formation by southern blotting

Samples from the DNA extracts were digested with EcoRI and NotI (New England Biolabs) for 1hr at 37°C. Equal DNA usage between samples was insured by basing the amount of digested DNA on the results of the control amplicon qPCR. The digested DNA was then separated on an 0.8% agarose gel and Southern transferred to Hybond N+ (GE Healthcare) as described by the manufacturer (NOTE: Hybond N+ is discontinued! Use Hybond XL instead!).

A 1.5kb PCR product corresponding to the region 1026-2532bp distal of the break at *ARS607::HOcs* was used for labelling. This PCR product was cloned into pGEM-T (Promega) to make pCZ29, and miniprep plasmid was used in PCR to generate template for labelling using the Prime-a-Gene system (Promega). Disappearance of a 4.3kb band corresponding to the intact locus was charted. Bands were quantified using Imagequant v.1.2 (Molecular Dynamics).

Mathematical calculations

In real-time PCR, relative differences in template DNA are calculated by comparing the number of PCR cycles required to reach a specific fluorescence level, known as threshold fluorescence. This value is referred to as threshold cycle, Ct, and the difference in Ct between two reactions is hence known as ΔCt. Each cycle difference between two reactions is a consequence of a two-fold difference in template amounts. Threshold fluorescence is set in the mid-range of exponential detection of PCR product formation.

Rather than doing the usual triplicates of identical reactions during qPCR, it was decided to use three serial dilutions of BstUI digests and mock digests (see above). This step controlled for the linearity of PCR reactions. After subtraction of one cycle per dilution, averages of the three Ct values were calculated.

The following formula describes the percentual difference in template amounts between two reactions:

$$\% \text{ difference} = 100/(2^{\Delta Ct}) \qquad \text{(Equation 1)}$$

In the specific case of the assay described here, ΔCt describes the difference in average Ct values between undigested and digested template. ΔCt is thus computed from the following equation:

$$\Delta Ct = Ct_{(+BstUI,\ break)} - Ct_{(-BstUI,\ break)} \qquad \text{(Equation 2)}$$

(where $Ct_{(+BstUI,\ break)}$ is the Ct value for a given amplicon at the break region using digested DNA as template, and $Ct_{(-BstUI,\ break)}$ is the Ct value for its mock-digested counterpart)

To accommodate possible differences in the input amounts between digested and undigested samples, ΔCt was normalised to the control amplicon (Table 2.4). In this equation, $Ct_{(+BstUI,\ control)}$ is the Ct value of the control amplicon using digested DNA as template, and $Ct_{(-BstUI,\ control)}$ is the Ct value for its mock-digested counterpart:

$$\Delta Ct = (Ct_{(+BstUI,\ break)} - Ct_{(-BstUI,\ break)}) - (Ct_{(+BstUI,\ control)} - Ct_{(-BstUI,\ control)}) \qquad \text{(Equation 3)}$$

Equation 1 only describes the difference in template amounts between two reactions. Since all the template DNA that remains after BstUI digestion is single-stranded, Equation 1 furthermore has to be modified as follows to describe the percentage of resected molecules at a given locus at a given time:

$$\% \text{ resected} = 100/[(1+2^{\Delta Ct})/2] \qquad \text{(Equation 4)}$$

Lastly, the fraction of molecules cut by HO (f) has to be taken into account:

$$\% \text{ resected} = \{100/[(1+2^{\Delta Ct})/2]\}/f \qquad \text{(Equation 5)}$$

The equations described above allow the calculation of the proportion of ssDNA as a percentage of the total DNA that is present at each time point. It is also possible to determine the number of resected molecules present at each time point (t_i) relative to the number of molecules that were present before HO induction (t_0). In this case, ΔCt is described by the following equation:

$$\Delta Ct=[(Ctt_{0(\text{-BstUI, control})}\text{-}Ctt_{i(\text{+BstUI, control})})+Ctt_{i(\text{+BstUI, break})}]\text{-}Ctt_{0(\text{-BstUI, break})}$$

(Equation 6)

Here, ΔCt describes the difference between undigested template at t_0 and digested template at a given time point t_i.

The percentage of ssDNA is then calculated by using following equation:

$$\%\text{resected}=(100/2^{\Delta Ct-1})/f$$ (Equation 7)

Lastly, information obtained from qPCR can also be used to quantify whole template levels for each amplicon throughout a time course. Equation 1 can be used to calculate template levels when ΔCt is expressed by following formula.

$$\Delta Ct=[(Ctt_{0(\text{-BstUI, control})}\text{-}Ctt_{i(\text{-BstUI, control})})+Ctt_{i(\text{-BstUI, break})}]\text{-}Ctt_{0(\text{-BstUI, break})}$$

(Equation 8)

Since the above section contains only the raw equations, the following paragraphs describe the derivation of the Equations by means of descriptive examples. It may be worth drawing out these examples for better understanding.

Derivation of the values of ssDNA relative to t_i

Consider the following 3 ideal cases of 100% cutting:

(1): 1/2 of the molecules in the sample are resected at a given amplicon. Because both strands are amplified in qPCR this means 1/3 of the DNA remains after BstUI digestion.

(2): 1/3 of the molecules in the sample are resected at a given amplicon. 1/5 of the DNA remains after BstUI digestion.

(3): 1/4 of the molecules in the sample are resected at a given amplicon. 1/7 of the DNA remains after BstUI digestion.

These examples show that you can describe the amount of template remaining as a mathematical row. Generalised, therefore, the actual amount of template that remains equals

$$1/(2x-1)$$ (Equation 9), where

x=1/fraction resected (Equation 10); e.g.: 1/4 of the molecules resected: 1/[2*(1/0.25)-1]=1/7

Equation 10 can be changed to the following format:

fraction resected=1/x (Equation 11)

The fraction that remains in qPCR can also be described as 1/(fold difference in PCR efficiency). Therefore,

1/(2x-1) = 1/(fold difference)

This can be changed to read

x = (1+fold difference)/2 (Equation 12)

Using Equation 12 to describe "x" in Equation 11, this therefore means

fraction resected=1/[(1+fold difference)/2] (Equation 13)

Since the fold difference between two PCR reactions is $2^{\Delta Ct}$, Equation 13 can be transformed to read

Fraction resected= $1/[(1+2^{\Delta Ct})/2]$ (Equation 14)

Equation 14 can be used to describe the percentage resected rather than the fraction resected:

% resected=$100/[(1+2^{\Delta Ct})/2]$, which is Equation 4. This equation can be divided by the fraction cut by HO to result in Equation 5, which describes the percentage of cut molecules that are resected, and corrects for the minor amount of uncut DNA present in each sample.

Derivation of the values of ssDNA relative to t_0

Again, consider three examples of the ideal case of 100% break formation first. Also, assume that the 3' strands are completely stable in these examples. Remember that there is no resection at t_0.

(1) 1/2 of the starting DNA molecules are resected at a given amplicon at a given timepoint t_i. Because both strands are amplified in qPCR, this means that 1/4 of the template DNA remains when you compare BstUI digested DNA at t_i with undigested DNA at t_0.

(2) 1/3 of the starting DNA molecules are resected at t_i. Therefore, 1/6 of the DNA remains after BstUI digestion when compared to undigested sample at t_0.

(3) 1/4 of the starting DNA molecules are resected at t_i. Therefore, 1/8 of the DNA remains after BstUI digestion when compared to undigested sample at t_0.

These examples show once again that the amount of template remaining can be described as a mathematical row. Generalised, the fraction of template that remains at a given amplicon after BstUI digestion of samples taken at t_i when compared to undigested template at t_0 equals $1/2^{\Delta Ct}$. As is obvious from the introduced examples, the fraction that is resected can therefore be described by the following term:

Fraction resected=$1/2^{\Delta Ct-1}$ (Equation 15), in the case of example (2): $1/2^{2-1}=1/2$

Therefore, the percentage of resected molecules can be described by

% resected=$100/2^{\Delta Ct-1}$ (Equation 16)

Division by the fraction of molecules cut by HO (f) results in Equation 7 (see above), and corrects for the small amount of uncut chromosomes. It is not necessary to correct for the amount of molecules whose 3' strand has been resected in addition to their 5' strand at a given amplicon as these will not contribute to the qPCR.

Table 1: Oligonucleotides used for qPCR.

Amplicon	Oligo	Sequence (5'-3')	Concentration
0.3kb	OCZ12	GGCGCGAAGCAAAAATTAC	400nM
	OCZ126	AAGAACCTCAGTGGCAAATCC	400nM
	OCZ140	FAM-TCCTCGCTGCAGACCTGCGA-TAMRA	150nM
9kb	OCZ127	GAAACCTCCTGCCGCCTT	600nM
	OCZ128	GTTGTAGCTGGCATCTCCTTATGT	600nM
	OCZ141	FAM-TCATCCTTCGACTTAGGGAAGAATCTTAACAAATG-TAMRA	200nM
14kb	OCZ129	ACCATACAACTTTCGCACGAC	600nM
	OCZ130	AAGGAAGTGTCTATGGACCGAAC	600nM
	OCZ142	FAM-TGATCATATCTTTGCAGAAAATAAACGAACCAAGAC-TAMRA	200nM
internal control, chromosome XIII	OCZ135	AATCAAATAGGCGTGGAGCA	400nM
	OCZ136	TTCGCTGTCTATCAACTCTAGATCAG	400nM
	OCZ139	VIC-TGCGTCCTTTTCCAGATCATCTTCCA-TAMRA	200nM