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The Fidelity Index provides a systematic quantitation of star activity of DNA restriction endonucleases

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ABSTRACT

Restriction endonucleases are the basic tools of molecular biology. Many restriction endonucleases show relaxed sequence recognition, called star activity, as an inherent property under various digestion conditions including the optimal ones. To quantify this property we propose the concept of the Fidelity Index (FI), which is defined as the ratio of the maximum enzyme amount showing no star activity to the minimum amount needed for complete digestion at the cognate recognition site for any particular restriction endonuclease. Fidelity indices for a large number of restriction endonucleases are reported here. The effects of reaction vessel, reaction volume, incubation mode, substrate differences, reaction time, reaction temperature and additional glycerol, DMSO, ethanol and Mn^{2+} on the FI are also investigated. The FI provides a practical guideline for the use of restriction endonucleases and defines a fundamental property by which restriction endonucleases can be characterized.

INTRODUCTION

Restriction endonucleases (REases) are enzymes that cleave double-stranded DNAs in a sequence-specific manner (1–4). They are ubiquitously present among prokaryotic organisms (5), where they form part of restriction-modification systems, which usually consist of an endonuclease and a methyltransferase. The cognate methyltransferase minimally methylates the same specific sequence that its paired endonuclease recognizes and renders the modified DNA resistant to cleavage by the endonuclease so that the host DNA can be properly protected. However, when there is an invasion of foreign DNA, in particular during bacteriophage infection, the foreign DNA will be degraded before it can be completely methylated. This is the major biological function of the restriction-modification system, protecting the host from bacteriophages (6). Other functions have also been suggested, such as involvement in recombination and transposition (7–9).

The nearly perfect specificity of the ~3000 known REases for their >250 different targets has been considered the key identifying feature during their characterization. After the discovery of the sequence-specific nature of the first REase (10,11), it did not take long for people to find that certain REases cleave sequences which were similar, but not identical, to their defined recognition sequences under nonoptimal conditions (12,13). This relaxed specificity was termed star activity. Many REases with star activity have been reported: AvaI (14), BamHI (15–20), BanI (21), Bme126I (22), BmrI (23), BspLU111III (24), BstI (25), BsuRI (26), BtsI (27), CeqI (28), CviJI (29,30), EcoRI (12,31–39), EcoRV (40–42), HaeIII (14), HhaI (20), HindIII (13,33,43), HinfI (44,45), KpnI (46), MamI (47), MboII (48), NcuI (49), NotI (50), PpiI (51), PstI (14,20,52), PvuII (17,43,53,54), RsrI (55,56), SacI (43), SalI (20), Sau3AI (57), SgrAI (58), SphI (59), SstI (20), TaqI (60–62), Tth111I (63) and XbaI (14,20). Experimentally, it has been found that the following general conditions may increase star activity: high glycerol concentration (>5% v/v) (18–20,34), high enzyme to DNA ratio (usually >100 U of enzyme per microgram of DNA) (12,17,20,34), low ionic strength (<25 mM salt) (12,17,20,34,35), high pH (>8.0) (41), the presence of organic solvents (such as DMSO, ethanol) (20,34,43) and substitution of Mg^{2+} with other divalent cations (Mn^{2+}, Co^{2+}) (15,33–35,64). It has been suggested that water-mediated interactions between the REase and DNA are the key differences between specific complexes and star complexes (17,38,65), but the actual situation may be even more complicated and remains to be clarified.

Star activity is not desirable for most REase applications; because it is normally weak and the sites of cleavage are not well defined, the products from star activity are much less predictable than the cognate activity. In conventional cloning, both the vector and the insert are

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cleaved by the same pair of REases, generating compatible ends for ligation. On the other hand, star activity can introduce undesirable cuts in the vector and the insert at extra sites perhaps destroying either one or both and leading to low yield of correctly ligated products. In cases such as forensic applications, where a certain DNA substrate needs to be cleaved by a REase to generate a unique fingerprint, star activity will destroy existing band(s) and generate new band(s), complicating the analysis. Avoiding star activity is also critical in applications such as strand displacement amplification (66) and serial analysis of gene expression (67). Though star activity can be significantly enhanced in many abnormal circumstances, for some enzymes, it can occur under normal reaction conditions. For those enzymes with apparent star activity, uncertainties in the substrate amount, substrate quality and the exact amount of restriction endonuclease can lead to unpredictable results. Many restriction endonucleases act differently toward different substrates; for example, while 1 U of a restriction endonuclease may be sufficient to cleave 1 μg of one specific substrate such as bacteriophage lambda DNA, it may not be the optimal amount for a different substrate. Furthermore, the activity of restriction endonucleases may change during storage and manipulation, and therefore reaction completeness and the effects of star activity may vary. For some enzymes, the restriction digestion reaction may not reach completion before star activity is observed.

Nevertheless, in some cases star activity is advantageous; in the years when there were limited numbers of REases available, certain REases were deliberately used under star conditions so that virtual new specificities were obtained. For example, EcoRI cleaves 5′-GAATTC-3′under normal conditions, and is reported to cleave 5′NAATTN-3′ under star conditions (12,68); when used under star conditions, EcoRI behaves like an enzyme that recognizes any 5′AATT-3′ sequence, which is the same as the newer enzyme Tsp509I. In another case, the star activity of BamHI was used to generate unidirectional deletion vectors (16).

Even though the concept of the star activity of a REase has been known for over 30 years, no systematic study on star activity has yet been published, largely due to the absence of a quantitative definition and the lack of sufficiently high concentrations of restriction endonucleases to allow a systematic analysis. Here we propose a quantitative definition of star activity, which we call the Fidelity Index (FI). It is the ratio of the highest amount of a REase showing no star activity during digestion (HNS) to the lowest amount needed for complete digestion on cognate sites (LCC). Similar to the unit definition of the restriction endonuclease, FI is measured on specific DNA substrates under specified conditions, mostly identical to the unit definition conditions. The factors that could alter the effective FI are also surveyed. It must be emphasized that while star activity is an intrinsic physical property of REases, it is not an all-or-none phenomenon and does not necessarily require unusual digestion conditions to be observed. For some REases, a very high concentration of the enzyme can exist in the reaction and still have no star activity, while with others, adding a slight excess or even the minimum amount to achieve complete digestion is enough to reveal star activity for others.

MATERIALS AND METHODS

FI determination assay

Most tested REases used were from New England Biolabs, Inc. (Ipswich, MA, USA) (NEB) as stock solutions of the highest available concentration, usually significantly higher than the commercially available standard concentration. Because of the need for high and accurate concentration of the enzymes, we compared only a few of them from other suppliers to confirm that our results are not an artifact of our preparation procedures. EcoRI and BamHI from the following REase suppliers were tested: Fermentas International Inc. (Burlington, Canada), GE Healthcare (Piscataway, NJ, USA), Invitrogen Corporation (Carlsbad, CA, USA), Promega Corporation (Madison, WI, USA), Roche Applied Science (Mannheim, Germany), Sibenzyme Ltd., (Novosibirsk, Russia), and Takara Bio Inc. (Shiga, Japan). HindIII, NotI and PstI from Fermentas International Inc. were also tested. Typically, to measure the FI, a concentrated REase stock solution was first subjected to a series of 2-fold dilutions using appropriate diluents, to give 20 different concentrations (1×, 0.5×, 0.25×, etc.). All of the diluents A, B and C contain 50% glycerol, like the starting REase stock solution. All reactions have the same glycerol concentration in the standard FI determination. The exact components for the diluents are as following: Diluent A: 50 mM KCl, 10 mM Tris–HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 200 μg/ml BSA, 50% glycerol (v/v, same throughout this paper); Diluent B: 300 mM NaCl, 10 mM Tris–HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 μg/ml BSA, 50% glycerol; Diluent C: 250 mM NaCl, 10 mM Tris–HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 200 μg/ml BSA, 50% glycerol (all diluents pH 7.4 at 25%). Three microliters of the enzyme solution were then mixed with 0.6 μg DNA substrate in a total reaction volume of 30 μl in each of the four following reaction buffers: N1: 10 mM Bis Tris Propane–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.0 at 25°C); N2: 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 at 25°C); N3: 100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 at 25°C); N4: 50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH7.9 at 25°C). Each commercial REase supplier has their own buffer system; however, many are comparable to the buffer systems tested here. The reactions were carried out in a 96-well microplate (Corning 3370 flat bottom assay plate with low evaporation lid). The 30 μl format was chosen for ease of reaction operation and gel-loading. All components were at the same concentrations as used for REase unit determination (which specifies a different volume: digestion of 1 μg DNA in a total volume of 50 μl). Reactions in the 30-μl format and 50-μl format were tested and shown to give identical results for the FI for EcoRI and BamHI. A 10% volume of restriction endonuclease was added, so that a final glycerol concentration of 5% was achieved. This glycerol

concentration was chosen because it is the highest suggested amount from most restriction endonuclease suppliers and represents the likely worst-case scenario in which to observe unwanted star activity. The plate was sealed by tape, covered with a lid and incubated in an air incubator at the temperature of the unit definition of the enzyme for 1 h before being quenched by stop solution (10×, 200 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.03% bromophenol blue, 0.94% SDS) and loaded onto an agarose gel. After electrophoresis, a picture of the gel was taken using a UV imager (Bio-Rad). The photograph was deliberately overexposed a little to reveal subtle weak bands; some bands will thus be saturated but the major bands were still clearly resolved. The gel picture was taken with a white background to increase contrast. For each set of reactions, the FI was calculated based on the dilution of the lane first showing no star activity compared to the last lane showing no partial digestion.

FIs for different substrates

BamHI was chosen as the test enzyme to compare the FIs obtained for different DNA substrates. For BamHI, the following substrates were chosen: λ, pBC4, pBR322, pMAL-c2E, pTWIN2, pUC19, pTWIN-MBP1, pXba, Litmus 28i and pGPS3 DNAs. The buffer N2 was chosen to provide the clearest demonstration of star activity. All substrates were from New England Biolabs, Inc. The complete sequences of each DNA are available from the NEBcutter homepage (http://tools.neb.com/NEBcutter2/index.php) (69). Each reaction contained 0.6 μg DNA in a total volume of 30 μl. The reactions were performed in 96-well plates at 37°C for 1 h.

Reaction temperature effect

A thermophilic REase, BstEII, was chosen for this study so that reactions could proceed at 60°C without inactivating the enzyme. The substrate was λ DNA and the buffer was N1. Reactions were carried out at 25, 30, 37 or 60°C to get the FIs at different temperatures.

The effect of external factors

λ DNA measuring 0.6 μg was digested by PstI in its optimal buffer, Buffer N3, at 37°C for 1 h, with 5% glycerol and with additional different concentrations of glycerol, DMSO, ethanol or $MnCl_2$, respectively. The FIs were measured individually under each of the different conditions.

The effect of supercoiled and linear forms of DNA on the FI

The same amounts of supercoiled or PstI-digested pBR322 were digested by BamHI, SspI, SphI, EcoRI and PvuII, and the FIs were compared for the supercoiled and linear substrates.

FIs of REases in PCR extension mixture

The FIs of REases have been determined in two PCR extension mixtures: three microliters of each REase were incubated at the appropriate reaction temperature for 1 h in a PCR mix containing 0.6 μg of DNA and 0.6 U of *Taq* DNA polymerase in a 30-μl reaction volume of 1× ThermoPol Buffer (10 mM KCl, 20 mM Tris–HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100), or 1× Standard *Taq* DNA Polymerase Buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM KCl, 1.5 mM $MgCl_2$, (pH 8.3 at 25°C), supplemented with dNTPs (200 μM final concentration). The following REases have been tested: AatII, BamHI, BglII, BsrGI, EcoRI, HaeIII, HindIII, MseI, NcoI, NdeI, NheI, NotI, SmaI, SpeI, SphI and XbaI.

RESULTS

The definition of FI

An example of a typical gel from an FI measurement experiment is shown in Figure 1. λ DNA is cleaved by ScaI in buffer N3. Each lane contains the same amount of DNA (0.6 μg unless stated otherwise). The amount of enzyme is reduced as a 2-fold serial dilution across the gel. At the far right, there is very little enzyme, so little or no DNA is digested. As the amount increases toward the left, partially digested fragments start to appear, which eventually become fully digested when the amount of the enzyme reaches a critical point. This we call the lowest REase amount needed for complete cleavage on cognate sites (LCC). At this point, all bands that can only be generated by this specific REase cleaving at its designated recognition sequence reach their highest intensities, and no other bands are present. This gives rise to the unique cleavage pattern that can be predicted based on the DNA sequence of the substrate and the recognition sequence of

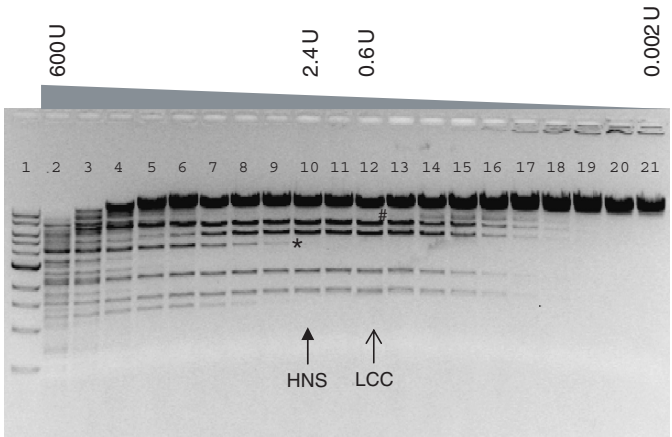

Figure 1. Definition of fidelity index. λ DNA is cleaved by ScaI in buffer N3. The enzyme concentration is reduced by a series of two fold dilutions in diluent A. Lane1: 0.5 μg NEB 1-kb DNA Marker; lane 2 contains 600 U; lane 10 contains 2.4 U; lane 12 contains 0.6 U; lane 21 contains 0.002 U ScaI. Reactions contain 3 μl of diluted ScaI, 1.2 μl λ DNA (0.6 μg), 3 μl buffer N3 and 22.8 μl of H_2O. The reaction is at 37°C for 1 h. The vertical arrows correspond to the two critical points: HNS—the Highest REases amount showing No Star activity and LCC—the Lowest REase amount needed for Complete Cleavage on cognate sites. FI = HNS/LCC, it is 4 in this case. Asterisk is a star activity band, and 'hash' is a partial activity band. This is also a representative example of the 'Star-prone' class REases.

the enzyme. This pattern remains as the REase amount continues increasing until the star activity of the enzyme begins to appear. The amount of the REase for the lane that immediately precedes the initial star activity lane will be considered the highest amount showing no star activity (HNS). Once star activity appears, the normal cleavage band(s) is further cleaved into smaller fragments, visible as a weakening of the normal band(s) and the appearance of a new star activity band(s).

We define the FI, which describes the degree of star activity for a REase, as the ratio of the highest REase amount showing no star activity (HNS) to the lowest REase amount needed for completed cleavage on cognate sites (LCC):

$$\mathrm{FI} = \frac{\mathrm{HNS}}{\mathrm{LCC}}$$

For the above example, the LCC is 0.6 U, because that is the minimum amount of enzyme needed to digest 0.6 μg DNA in 1 h. The HNS is 2.4 U because above that amount additional bands begin to appear due to star activity. Thus the FI of ScaI in N3 is 2.4/0.6 = 4. The FI is an indicator of how specific an enzyme is. The higher the number, the greater the specificity of the enzyme and the larger the window of enzyme concentration that can be used without the deleterious consequences of star activity.

The FIs for selected REases

Because an accurate determination of the FI often requires high enzyme concentration, only 213 enzymes were available at suitable concentrations. The FIs of these 213 REases in four buffers (N1–N4) were measured. The FIs for the 56 most representative REases are listed in Table 1. The comparison of the LCC lanes in each buffer also indicates the relative activity of the REase in various buffers. The relative activity in the best buffer is designated as 100%, and those in the other buffers are the relative activity percentage numbers. It should be emphasized that the FIs listed in Table 1 are all determined under normal reaction conditions, i.e. DNA was at 20 ng/μl, REase was at 10% of the volume and reaction was at the recommended temperature for 1 h. Compared to the conditions for the activity unit determination, the conditions for the FI determination are the same except for the selection of substrate in some cases. When there are too many bands (such as the 4-base REase MspI on λ DNA) the extra DNA bands caused by star activity can be difficult to observe. On the other hand, when the substrate is relatively short, such as when the small plasmid pNEB193 is used as the substrate in the unit definition for the 8-base REase PacI, the sequence complexity of the substrate may be too low to contain star activity sites, so that star activity cannot be detected. In both cases, a suitable substrate of appropriate size was selected to demonstrate both cognate activity and star activity.

Because of the way in which the FI determination assay was designed, all FIs have values of 2^n, with n being a positive or negative integer. For convenience, the FIs were recorded as 0.02, 0.06, 0.13, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 120, 250, 500, 1000, 2000, 4000, 8000, 16 000, 32 000, 64 000. FIs for different REases in different buffers varied from as small as 0.02 (ScaI in buffer N1) to as large as ≥64 000 (HaeIII in buffer N2). When no star activity was seen even at the highest enzyme concentration tested, a greater-than-or-equal-to number is given.

FIs of 500 and above (Figure 2A) indicate that the REase can be used with as much as a 500-fold over-digestion in that buffer. An FI of 120 or 250 (Figure 2B) indicates that there is a good range of REase concentrations that do not lead to visible star activity while the reaction is still complete. An FI of 64 or less suggests that caution should be used because the REase may show apparent star activity in the tested buffer. Often digestions are allowed to proceed for more than 1 h, which will worsen the situation if star activity is present. In some cases, the FI can be <1, which means that the star activity bands appear even before the normal complete digestion pattern is established.

Some FIs require special consideration. For instance, a low FI may be caused by a rather low relative activity at canonical sites, such as AscI in buffer N1. Here, the FI is 8 but the activity relative to the best buffer is only 0.1% and as a result is labeled as 0%. Some REases also cannot complete the reaction at all in certain buffers, no matter how much is added; here the FI is listed as NC (Not Complete). Practically speaking, an REase will never be recommended for use in a buffer in either of these two cases. Some FIs are inconclusive numbers, which are greater than or equal to a number that is no more than 64, such as ≥1, ≥2, ≥4, ≥8, ≥16, ≥32, ≥64. The actual FI can be high yet can also be low. This happens because the available REase concentration is too low to find the concentration that exhibits star activity. This can only be resolved when a higher starting concentration of the REase becomes available. The FIs in the above three cases are not used for the further classification as described below.

Based on the FIs in all four tested buffers, the enzymes can be categorized into four different classes depending on their different behaviors in the tested buffers. The four classes are described below.

'Excellent'. At least one FI is at least 500 and all FIs are at least 120. For this class, there is at least one buffer in which little or no star activity is present and there is no buffer in which severe star activity is seen. Eighty-three REases fall into this class. The cleavage pattern of one member of this class, MspI, is shown in Figure 2A. No star activity was seen even at the highest REase amount (9600 U).

'Good'. All FIs are either 120 or 250. Here at least one buffer can be used without having significant star activity, and still there is no buffer that will cause severe star activity. Twenty-two REases are in this class. A representative example (BstBI) is shown in Figure 2B.

'Star-prone'. At least one FI is no more than 64 and no FI is more than 250. The enzymes from this class have traditionally been considered to display star activity

Table 1. Selected list of Fidelity Indices of restriction endonucleases

Enzyme	Substrate DNA	N1	N2	N3	N4	Class
AatII	λ	NC	$\geq 32^{3}$	NC	$\geq 500^{100}$	Excellent
AciI	pXba	$\geq 120^{50}$	$\geq 250^{100}$	$\geq 120^{50}$	$\geq 120^{50}$	Good
AgeI	pXba	16^{100}	8^{50}	64^{13}	8^{50}	Star-prone
ApaI	pXba	$\geq 250^{13}$	$\geq 250^{13}$	$\geq 4^{0}$	$\geq 2000^{100}$	Excellent
AscI	λ	8^{0}	4000^{50}	250^{3}	4000^{100}	Excellent
AvrII	T7	64^{100}	8^{100}	32^{25}	32^{100}	Star-prone
BamHI	λ	4^{50}	4^{100}	32^{100}	8^{50}	Star-prone
BglII	pXba	2000^{13}	2000^{25}	250^{100}	250^{13}	Excellent
BsrGI	λ	64^{6}	250^{100}	1000^{100}	120^{25}	Excellent
BstBI	λ	250^{50}	250^{50}	$\geq 64^{1}$	250^{100}	Good
BstEII	λ	16^{50}	4^{100}	16^{50}	4^{100}	Star-prone
ClaI	dam⁻ λ	500^{50}	500^{100}	120^{25}	250^{100}	Excellent
DdeI	pBR322	$\geq 2000^{100}$	$\geq 8000^{50}$	$\geq 1000^{6}$	$\geq 1000^{50}$	Excellent
DpnI	pBR322	$\geq 250^{100}$	$\geq 1000^{50}$	$\geq 2000^{25}$	$\geq 1000^{100}$	Excellent
EagI	pXba	4^{25}	8^{50}	250^{100}	16^{100}	Star-prone
EcoRI	λ	250^{50}	4^{100}	250^{100}	4^{100}	Star-prone
EcoRV	pXba	32^{6}	120^{50}	1000^{100}	64^{25}	Variable
FseI	Adenovirus-2	$\geq 120^{100}$	$\geq 16^{13}$	$\geq 2^{2}$	$\geq 64^{50}$	Good
HaeIII	pBR322	$\geq 2000^{50}$	$\geq 64\,000^{100}$	$\geq 16\,000^{25}$	$\geq 32\,000^{50}$	Excellent
HindIII	λ	32^{25}	250^{100}	4000^{25}	32^{50}	Variable
HpaI	λ	32^{6}	1^{25}	2^{13}	16^{100}	Star-prone
KpnI	pXba	16^{100}	16^{25}	8^{6}	4^{50}	Star-prone
MboII	dam⁻ λ	4^{50}	16^{50}	64^{50}	16^{100}	Star-prone
MfeI	λ	32^{100}	16^{13}	8^{6}	32^{100}	Star-prone
MluI	λ	1000^{13}	120^{25}	4000^{100}	250^{13}	Excellent
MscI	pBC4	$\geq 8000^{25}$	8000^{50}	1000^{25}	1000^{100}	Excellent
MseI	pBR322	$\geq 120^{100}$	$\geq 120^{100}$	$\geq 120^{100}$	$\geq 120^{100}$	Good
MspI	pBR322	$\geq 16\,000^{50}$	$\geq 16\,000^{100}$	$\geq 8000^{25}$	$\geq 32\,000^{100}$	Excellent
NcoI	λ	120^{100}	32^{100}	120^{25}	32^{100}	Star-prone
NdeI	λ	1000^{50}	1000^{100}	8000^{100}	1000^{50}	Excellent
NheI	pXba	32^{100}	120^{25}	120^{13}	32^{100}	Star-prone
NlaIII	ΦX174	$\geq 32^{0}$	$\geq 32^{0}$	$\geq 8^{0}$	4000^{100}	Excellent
NotI	pXba	$\geq 2000^{6}$	64^{100}	500^{100}	32^{25}	Variable
NspI	pBR322	$\geq 2000^{100}$	$\geq 32^{2}$	NC	$\geq 500^{25}$	Excellent
PacI	T7	500^{100}	120^{25}	120^{3}	1000^{100}	Excellent
PmeI	Adenovirus-2	500^{13}	2000^{13}	$\geq 2000^{1}$	500^{100}	Excellent
PstI	λ	64^{100}	32^{100}	120^{100}	8^{50}	Star-prone
PvuI	λ	250^{6}	1000^{25}	4000^{100}	1000^{25}	Excellent
PvuII	pBR322	250^{100}	16^{25}	8^{3}	0.25^{100}	Star-prone
RsaI	pBR322	$\geq 2000^{100}$	$\geq 1000^{50}$	$\geq 32^{2}$	$\geq 2000^{100}$	Excellent
SacI	pXba	120^{100}	120^{50}	120^{3}	32^{50}	Star-prone
SacII	pXba	2000^{13}	1000^{50}	$\geq 120^{1}$	1000^{100}	Excellent
SalI	HindIII digestedλ	8^{0}	16^{6}	32^{100}	1^{1}	Star-prone
SapI	λ	16^{25}	64^{50}	32^{25}	16^{100}	Star-prone
ScaI	λ	0.06^{3}	0.13^{100}	4^{50}	0.02^{6}	Star-prone
SfiI	pXba	1000^{100}	8000^{100}	1000^{50}	4000^{50}	Excellent
SmaI	pXba	$\geq 16^{2}$	$\geq 64^{6}$	$\geq 8^{0}$	1000^{100}	Excellent
SpeI	pXba	500^{100}	500^{100}	500^{6}	500^{100}	Excellent
SphI	λ	64^{100}	32^{100}	64^{25}	16^{50}	Star-prone
SspI	λ	64^{100}	16^{100}	32^{25}	16^{100}	Star-prone
StuI	dam⁻ λ	1000^{50}	500^{50}	1000^{50}	4000^{100}	Excellent
StyI	λ	32^{25}	16^{100}	32^{50}	16^{25}	Star-prone
XbaI	pXba	$\geq 8000^{25}$	$\geq 32\,000^{100}$	$\geq 4000^{13}$	$\geq 16\,000^{50}$	Excellent
XhoI	pXba	$\geq 4000^{25}$	$\geq 8000^{100}$	$\geq 8000^{100}$	$\geq 8000^{100}$	Excellent
XmaI	pXba	8^{50}	4^{50}	NC	1^{100}	Star-prone
XmnI	λ	$\geq 8000^{100}$	$\geq 4000^{50}$	$\geq 500^{6}$	$\geq 4000^{50}$	Excellent

N1, N2, N3, and N4: Fidelity indices for the tested buffers, the superscript is the percentage activity relative to the optimal buffer.
Class: Enzyme Class: 'Excellent','Good','Star-prone'and 'Variable'—see the results for definitions.
NC: Incomplete digestion up to the highest amount of REase tested.

(ScaI, Figure 1). The basic reason why this cut-off range at 250 is selected is that it applies to the 'classic' example of star activity, EcoRI, which has an FI of 250 in buffers N1 and N3 and an FI of 4 in buffers N2 and N4. Ninety-eight of the studied enzymes belong to this Class.

'Variable'. At least one FI is ≥500, while at least one FI is no more than 64. These REases can be used in some buffers with little or no star activity and in another buffer with significant star activity. Ten REases are of this type (NotI, Figure 3).

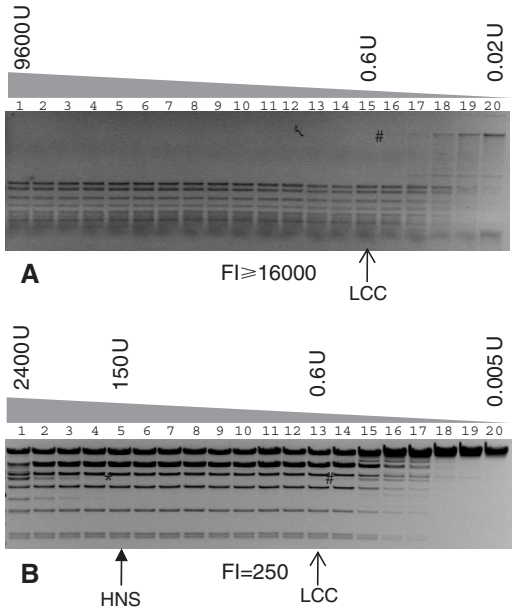

Figure 2. Representative examples of the 'Excellent' and 'Good' classes of REases in terms of star activity. (**A**) REase in the 'Excellent' class. Cleavage of pBR322 by MspI in buffer N1: MspI is 2-fold serially diluted with diluent A. The reaction contains 3 µl of diluted MspI, 0.6 µl pBR322(0.6 µg), 3 µl buffer N1 and 23.4 µl of H_2O. Reaction is at 37°C for 1 h. Lane 1: 9600 U MspI; lane 15: 0.6 U MspI (LCC); lane 20: 0.02 U MspI. FI ≥ 16 000. 'Hash'is a partial activity band, and there is no star activity band. (**B**) REase in 'Good' Class. Cleavage of λ DNA by BstBI in buffer N2. BstBI is 2-fold serially diluted with diluent A. The reaction contains 3 µl of diluted BstBI, 1.2 µl λ DNA (0.6 µg), 3 µl buffer N2 and 22.8 µl of H_2O. The reaction is at 65°C for 1 h. Lane 1: 2400 U BstBI; lane 5: 150 U BstBI (HNS); lane 13: 0.6 U BstBI (LCC); lane 20: 0.005 U BstBI, FI = 250. Asterisk is a star activity band, and 'hash' is a partial activity band.

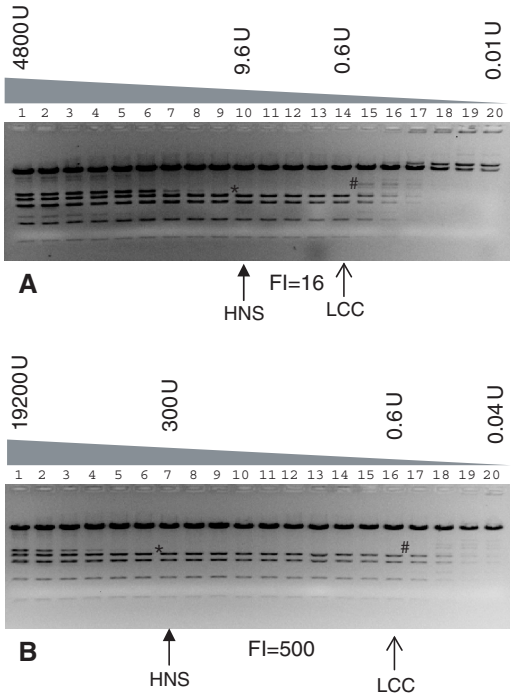

Figure 3. Representative example of the 'Variable' class of REase. Comparison of FIs of NotI in buffer N4 (**A**) and buffer N3 (**B**) using pXba DNA as the substrate. NotI is 2-fold serially diluted with diluent C. The reaction contains 3 µl of diluted NotI, 1.2 µl pXba DNA (0.6 µg), 3 µl buffer N4 (A) or buffer N3 (B) and 22.8 µl of H_2O. The reaction is at 37°C for 1 h. Asterisk is a star activity band, and 'hash' is a partial activity band. (**A**) Lane 1: 4800 U NotI; lane 10: 9.6 U NotI (HNS); lane 14: 0.6 U NotI (LCC); lane 20: 0.01 U, NotI; FI = 16. (**B**) Lane 1: 19 200 U; lane 7: 300 U (HNS); lane 16: 0.6 U (LCC); lane 20: 0.04 U. FI = 500.

The FI of REases is independent of the supplier

The same REases from different commercial suppliers were compared. EcoRI and BamHI from eight different companies as listed in the 'Materials and methods' section were all tested in the same buffers N2 and N4. These two REases were selected because not only are they well-known REases with star activity, but also that they are readily available at concentrations sufficient to show a range of star activity. Since the FIs comprise a set of values of 2^n, the designation of the partial activity band and the star activity band could be off by one lane depending on the starting REase concentration. So a variation of 2- to 4-fold is considered to be identical within experimental error. The FIs were essentially the same for EcoRI and BamHI from different suppliers (Table 2). Additionally, HindIII, NotI and PstI from Fermentas International Inc. were also checked in buffers N2 and N4, and the values were found to be similar to ones from NEB. While it would be desirable to test every REase from each REase suppliers, the availability of high concentration REases from suppliers other than NEB is limited. Our limited tests confirm that star activity is an intrinsic property of the REase and is independent of supplier. However, because the FI can vary a lot in the different buffers, the exact FI in the buffer system from other suppliers remains to be determined.

Table 2. FI variation from different commercial sources

REase suppliers	BamHI		EcoRI	
	N2	N4	N2	N4
Fermentas International Inc.	4	8	4	4
GE Healthcare	4	8	4	4
Invitrogen Corporation	8	8	8	4
New England Biolabs, Inc.	4	8	4	4
Promega Corporation	8	8	4	2
Roche Applied Science	8	8	8	4
Sibenzyme Ltd.	8	8	4	4
Takara Bio Inc.	8	8	8	4

The FIs of EcoRI and BamHI from 8 different suppliers in buffer N2 and N4.

Star activity is site specific

While it has been shown by many groups that star sites for a few enzymes are different from nonspecific sites, i.e. star sites have a certain specificity and are not random (12–14,18–20,33–35,41), this is the first time that star activity has been studied on such a large scale. Since star activity results from cleavage at specific secondary sites and is observed as extra discrete and unique band(s) other than the ones from the cognate sites, a cautionary note is necessary. This is illustrated in Figure 4 for NotI.

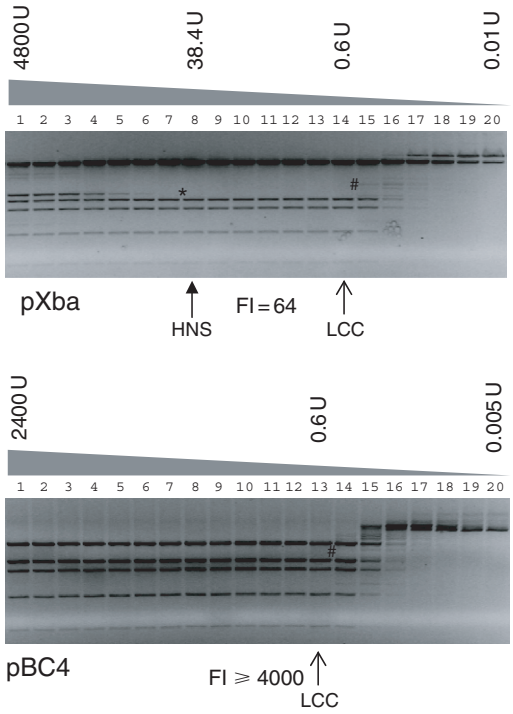

Figure 4. Star activity is site-specific. While a clear star activity band is seen for NotI when pXba is used as the substrate, no star activity is observed when pBC4 is used. Asterisk is a star activity band, and hash is a partial activity band in every panel. NotI is 2-fold serially diluted with diluent C. The reaction contains 3 µl of diluted NotI, 1.2 µl pXba or pBC4 (0.6 µg), 3 µl buffer N2 and 22.8 µl of H_2O. Reaction is at 37°C for 1 h. Upper panel: Lane 1: 4800 U NotI; lane 8: 38.4 U NotI (HNS); lane 14: 0.6 U NotI (LCC); lane 20: 0.01 U; FI = 64. Bottom Panel: Lane 1: 2400 U NotI; lane 13: 0.6 U NotI (LCC); lane 20: 0.005 U; FI ≥ 4000-.

While clear star activity bands were seen when pXba (22 kb) was used as the substrate, no star activity was seen with pBC4 (11 kb, a subset of pXba). The reason is that the major star activity site of NotI (ACGGCCGC) (50) is present in pXba but not in pBC4. Theoretically, for star activity, one positive result is sufficient to prove the REase has star activity, while even many negative results from different substrates can only suggest that the tested REase has no apparent star activity.

FI varies in different reaction buffers

Just like the normal cleavage activity of a REase, its star activity also varies from one buffer to another. Enzymes of the Variable class in Table 1 are extreme cases where there could be as great as a 2000-fold difference for FIs in different buffers. There are also a significant number of enzymes that have very similar FIs in all four different buffers. For all the enzymes studied, the percentages of enzymes that show the highest FIs in N1, N2, N3 and N4 are 24, 24, 24 and 28%, respectively. So generally speaking, there is no buffer that is much better than the others in terms of giving much less star activity. This is in total contrast to the normal cleavage activity of REases, where 41% of the REases are most active in buffer N4, followed by 25% in N2, 20% in N1 and 14% in N3. REases are ideally used in their optimal buffers; however, restriction digestions that include two or more REases with different optimal buffers are very common. This is why a selection of four reaction buffers instead of one was tested in this study.

Re-classification of the REase class

Through this study, we have found some REases which have previously been flagged as having star activity to actually be in the 'Excellent' or 'Good' Classes of REases, and *vice versa*. Enzymes of the former group include AvaI, BsoBI, BssHII, BstZ17I, HpyCh4III, NciI, PflFI, TfiI, XmnI and ZraI. The reason for the earlier categorization of these enzymes as possessing star activity is probably because the previous testing conditions are somewhat different from this study, such as with higher glycerol or longer incubation times. Fifty-seven REases, such as AgeI, previously considered as very specific are now shown to have star activity. Insufficient investigation had previously failed to detect and confirm the star activity of these REases.

FI variation on different substrates

It is well known that REases cleave different substrates with varying efficiency, because each individual substrate will have a different arrangement of REase sites, and each site will lie in a different sequence context which is expected to affect the digestion rate. Since the star activity is also a cleavage activity with specificity, the star activity will also vary on different substrates and will lead to unique star activity band(s). Thus, we expect that the FI will vary on different substrates.

For BamHI, different substrates gave different FIs in the same buffer N2. While BamHI has the cognate site, GGATCC, one group of major star sites for BamHI is GGNTCC (19) under normal reaction conditions, which can be greatly enhanced in the presence of glycerol. pMAL-c2E and pGPS3 had an FI of 1, while many substrates gave an FI of 2, such as pTWIN-2, pUC19, pTWIN-MBP1 and LITMUS28i. The FIs for λ DNA, pBR322 and pXba are 4. pBC4 had the highest FI of 8.

From this example of BamHI, an FI can vary up to 10-fold, even higher in some extreme cases. Although the FIs for different REases are measured on different substrates, the general trend is maintained, i.e., for a group of substrates, an enzyme from the 'Excellent' class is normally better than an enzyme from the 'Star-prone' class.

FI varies with reaction temperature

The digestion of λ DNA by BstEII was studied at different temperatures. The FIs for 25, 30, 37 and 60°C are ≥64, 32, 32 and 16, respectively. It was found that while a higher temperature slightly increased the normal cleavage activity of this enzyme, it increased the star activity to a larger extent. This was responsible for the decreased FI at the higher temperatures. Thus it is probably advisable to use temperatures in the lower range to ensure fidelity of cleavage and minimize star activity. BsoBI, a REase which has higher activity at 65°C, is most appropriately used at 37°C to minimize the star activity. The FIs of BamHI and

Table 3. The effects of additives on the FI of PstI

Glycerol (%)	FI	DMSO (%)	FI	Ethanol (%)	FI	$MnCl_2$ (mM)	FI
5	120	0	120	0	120	0	120
10	64	5	16	2	64	0.1	<1
15	32	10	8	4	64	0.2	<1
20	16	15	4	6	32	0.4	<1
25	8	20	1	8	16	0.8	<1
30	8	25	1	10	8	1.6	<1
35	4	30	<1	12	4	3.2	<1
43	4	35	<1	14	4	6.4	<1

The standard reaction condition contains 5% glycerol, buffer N3 and uses λ DNA as substrate. Incubation is at 37°C for 1 h. Each pair of columns presents the results for the single addition of glycerol, DMSO, Ethanol or $MnCl_2$ as indicated.

OkrAI at room temperature have been found to be about 10 times higher than that at 37°C (Xu,S.-Y., unpublished data).

Effects of additional external factors

It has been shown experimentally that the star activity of a REase can be enhanced by additional factors such as organic solvents or unusual metal ions (18–20, 33–35,41,43,64). Since the FI is a quantitative measurement of star activity, it is also altered by these additional factors. The FIs of PstI in buffer N3 on λ DNA, with different additional factors, are listed in Table 3. Increased amount of glycerol, DMSO, ethanol or $MnCl_2$ all reduce the FI significantly.

REases have the same FI on supercoiled and linearized DNA

Some DNAs are typically available in supercoiled form, while others are usually in linear form. We tested whether the differences between these two forms had an effect on the FI. The FIs of BamHI, EcoRI, PvuII, SphI and SspI on supercoiled pBR322 and PstI-linearized pBR322 were compared and found to be the same. The REase amounts needed for the HNS and the LCC are also essentially the same (data not shown).

FI and relative activity varies much in the PCR extension mixture

The FIs and relative activities of 16 REases have been determined in two PCR extension mixtures. The data are listed in Table 4. The two PCR extension mixtures represent two different buffer conditions, which have a higher pH than normal reaction buffers. Some REases perform well in the two PCR extension mixtures, such as AatII, HaeIII, HindIII, MseI, NheI and XbaI. Others either have much less activity such as BglII, NdeI or a much lower FI such as BamHI, EcoRI, NcoI, NotI, SmaI, SpeI and SphI. Occasionally, both relative activity and FI are less than in the normal reaction buffer. BsrGI is one such example. Unless a REase is specifically assayed in a PCR extension mixture, it seems unwise to assume that the FI will not be affected.

Table 4. FIs in the PCR extension mixture

	Taq polymerase buffer	ThermoPol buffer	(N1, N2, N3, N4)
AatII	$\geq 500^{100}$	$\geq 500^{100}$	$\geq 500^{100}$, N4
BamHI	4^{200}	4^{100}	32^{100}, N3
BglII	$\geq 32\,000^{25}$	$\geq 8000^{6}$	250^{100}, N3
BsrGI	8^{2}	32^{13}	1000^{100}, N3
EcoRI	8^{100}	1^{50}	250^{100}, N3
HaeIII	$\geq 1\,20\,000^{400}$	$\geq 1\,20\,000^{400}$	$\geq 64000^{100}$, N2
HindIII	8000^{200}	2000^{100}	250^{100}, N2
MseI	$\geq 120^{100}$	$\geq 64^{50}$	$\geq 120^{100}$, N2
NcoI	4^{100}	8^{100}	120^{100}, N1
NdeI	$\geq 2000^{100}$	$\geq 500^{25}$	8000^{100}, N3
NheI	1000^{100}	500^{100}	32^{100}, N4
NotI	120^{50}	64^{100}	500^{100}, N3
SmaI	64^{200}	250^{200}	1000^{100}, 4
SpeI	120^{50}	120^{100}	500^{100}, N2
SphI	8^{100}	4^{50}	64^{100}, N1
XbaI	2000^{100}	$\geq 16\,000^{100}$	$\geq 32\,000^{100}$, N2

FIs are listed as in the standard Taq polymerase buffer, ThermoPol buffer and compared to the FI measured in an optimum buffer (N1, N2, N3 and N4) as indicated.
All the substrates are same as in the Table 1.

DISCUSSION

We present here a quantitative measure of star activity and introduce the concept of a FI. Every REase has its own FI in each specific buffer. The FI can provide general guidelines for choosing the optimal REase and reaction conditions under which to use REases. Enzymes can be selected with little or no star activity over those that have strong star activity. This enables substrate digestion with a large excess of enzyme to ensure complete cleavage. On the other hand, if choices are limited and an enzyme with star activity must be used, the FI can help in choosing the reaction conditions needed to obtain complete cleavage without causing severe star activity. Just as in the case of double digestion, not only should the relative activity be considered, but also the FI. Often it will be more important to select reaction conditions that increase the fidelity of cleavage (i.e., high FI) than to aim for higher activity. For example, for a double digestion of BamHI and SphI, in buffer N2, the relative activity is 100% for both of the enzymes, but the FI is only 4 for BamHI and 32 for SphI. Buffer N3 is a better choice since BamHI has an FI of 32 and SphI has a FI of 64, which is much better for the inhibition of star activity.

The actual outcome of the reaction is also dependent on the reaction vessel, incubation time and mode. Four types of reaction vessels are common, including 1.7-ml microcentrifuge tubes, 0.5-ml microcentrifuge tubes, 0.2-ml PCR tubes and 96-well microplates. The 96-well plate is becoming more common as high-throughput applications appear. Though the reaction vessels are typically sealed during incubation, the air in the vessel will typically contain more water vapor during incubation than the air in the open space at room temperature when the reaction is set up—water will evaporate from the reaction. This will be enhanced with a larger vessel, or a smaller reaction volume or a higher reaction temperature. Since the concentrations of REase, DNA and buffer components are all

increased under these conditions, the star activity can become more severe. Significant water relocation may result if the vessel is not evenly heated, such as during water bath incubation or on a hot plate. For the same vessel and temperature, air incubation has been observed to have less evaporation than that water bath or hot plate. To minimize the star activity, reactions should be carried out in an air incubator, a PCR machine which can heat the reaction vessel evenly, or mineral oil should be used to prevent evaporation. Water baths and hot plates are only recommended if the reaction time is short such as 15 min and/or the starting reaction volume is relatively large to the reaction vessel ($\geq$100 µl for a 1.7-ml micro-centrifuge tube).

Enzymes have different FI numbers on different substrates. Unless the specific substrate has been tested, the worst-case scenario (i.e. lowest observed FI) should be assumed. When very low backgrounds are required (<0.1% of uncut, for example, in the digestion of a vector for cloning into), the amount of enzyme (in units) up to the FI can be used; when the detection limit is higher, such as gel-based fingerprinting, lesser amounts of enzyme should be used as long as complete digestion can be achieved.

Besides the immediate application of these findings to guide REase digestion, our definition also lays the foundation upon which more research can be done on the subject of star activity itself. Examples would include studies of the molecular basis of star activity, or the effects of additional external factors on star activity.

While determining FIs for each REase, we have discovered that it is possible to increase the FI of a number of REases by mutation, including BamHI and EcoRI. The mutants we have isolated typically retain the cognate activity of the wild type, but have considerably less star activity. This work will be the subject of a future publication.

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