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The pUltra plasmid series: a robust and flexible tool for fluorescent labeling of Enterobacteria

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Abstract

Fluorescent labeling has been an invaluable tool for the study of living organisms and bacterial species are no exception to this. Here we present and characterize the pUltra plasmids which express constitutively a fluorescent protein gene (GFP, RFP, YFP or CFP) from a strong synthetic promoter and are suitable for the fluorescent labeling of a broad range of Enterobacteria. The amount of expressed fluorophore from these genetic constructs is such, that the contours of the cells can be delineated on the basis of the fluorescent signal only. In addition, labeling through the pUltra plasmids can be used successfully for fluorescence and confocal microscopy while unambiguous distinction of cells labeled with different colors can be carried out efficiently by microscopy or flow cytometry. We compare the labeling provided by the pUltra plasmids with that of another plasmid series encoding fluorescent proteins and we show that the pUltra constructs are vastly superior in signal intensity and discrimination power without having any detectable growth rate effects for the bacterial population. We also use the pUltra plasmids to produce mixtures of differentially labeled pathogenic *Escherichia*, *Shigella* and *Salmonella* species which we test during infection of mammalian cells. We find that even inside the host cell, different strains can be distinguished effortlessly based on their fluorescence. We, therefore, conclude that the pUltra plasmids are a powerful labeling tool especially useful for complex biological experiments such as the visualization of ecosystems of different bacterial species or of enteric pathogens in contact with their hosts.

**Keywords:** fluorescent labeling, GFP, microscopy, Enterobacteria, pathogens
**Abbreviations:** GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; optical density $A_{600}$, OD600; Dulbecco’s Modified Eagle Media, DMEM; multiplicity of infection, MOI; enterohaemorrhagic *Escherichia coli*, EHEC; para-formaldehyde, PFA
1. Introduction

The discovery of the green fluorescent protein (GFP) in 1961 (1), its crystallization twelve years later (2) and the first production of fluorescence *in vivo* from a genetically engineered construct ten years after that (3) marked the beginning of a new era for cell biology, biochemistry and even ecology. Since its discovery, GFP has been modified to produce a plethora of differently colored variants (4), fusion proteins (3) and biosensors (5) which have been used in a wide spectrum of applications. Microbiology is one of many fields that has reaped the benefits of these new tools. Fluorescent tagging of proteins has allowed the investigation of the function and assembly of the most impressive molecular machines in bacteria (for instance protein translocation systems (6), cellular appendages (7-9) and toxin secretion structures (10-12)) while fluorescent labeling of the entire bacterial cell has led to impressive results in the study of naturally evolving bacterial populations (13,14) and of pathogenic bacteria in interaction with their hosts (15,16).

The small size and high division rate of bacteria enforce the use of fluorescent proteins that are particularly bright and mature rapidly following expression (17). For this reason, numerous application-specific fluorescent tools have been developed (3,18,19). These are often specialized constructs that have been optimized for certain bacterial species and microscopy setups, and have been engineered using only one of two types of fluorescent protein. As a result, these constructs are likely to have limited utility in the study of bacterial communities or pathogenic bacteria. In parallel, series of mini-Tn7 vectors expressing an array of fluorescent proteins which can be used in a broad range of bacterial species have also been developed (13,14,20,21). The integration of these constructs at a conserved site in the chromosome (specifically at the 3’ end of the *glmS* gene) has the advantage that the
fluorescent protein is expressed as a single-copy gene, ensuring that the entire population is labeled uniformly (20,21). However, the expression levels of the fluorescent proteins are relatively low and often lead to insufficient signal, especially for red fluorescent proteins (21), while disruptive insertions of Tn7 transposons at ectopic sites are also known to occur (20,22).

During this era of antibiotic resistance and superbug prevalence, the majority of research conducted on pathogenic Enterobacteria is related to their interactions with their host organisms (for example (15,16)). These studies require labeling of bacteria with bright and stable fluorescent proteins, expressed in large amounts by an easily transferable genetic construct (23,24). Ideally, one should be able to unambiguously distinguish between differently colored bacteria in the presence or absence of host cells, often for a length of time. Here we present and characterize the pUltra plasmids, a series of plasmids suitable for most Enterobacteria, where the fluorescent protein gene is expressed under the control of a strong synthetic promoter guaranteeing the constitutive production of the fluorescent protein in large amounts in the entire bacterial population. We have constructed pUltra plasmids that can express four different fluorescent proteins and we show that they can be used successfully in microscopy and flow cytometry experiments for the study of enteric bacteria, including pathogenic species.

2. Materials and Methods

2.1. Construction of plasmids
Plasmids and oligonucleotides used in this study are listed in Tables S1 and S2, respectively. KOD Hot Start DNA polymerase (Novagen, Madison, Wisconsin, United States) was used for PCRs according to manufacturer’s instructions, oligonucleotides were synthesized by IDT (Leuven, Belgium) and all constructs were sequenced and confirmed to be correct before use.

For the generation of pUltraYFP-KM and pUltraCFP-KM the yfp and cfp genes were amplified from pTn7YFP and pTn7CFP (21), respectively using primers P1 and P2. The strong synthetic Biofab promoter sequence (a modified version of the P2 (apFAB45) promoter, described as [PT7A1 (-35 region) + NM (linker) + PT525 (-10 region)] in (25)) of pUltraRFP-KM was included in the P1 primer. The yfp and cfp inserts were subsequently cloned into the EcoRI-BamHI sites of pUltraRFP-KM. For the generation of pUltraGFP-GM, pUltraRFP-GM, pUltraYFP-GM, and pUltraCFP-GM, the gentamicin cassette of pTn7YFP was amplified using primers P3 and P4 and cloned into the NruI site of pUltraGFP-KM, pUltraRFP-KM, pUltraYFP-KM, and pUltraCFP-KM. Since the original kanamycin cassette was disrupted in the construction process, the pUltra-GM plasmids only confer resistance to gentamicin.

2.2. Bacterial Strains and growth conditions

Bacterial strains used in this study are listed in Table S3. Plasmids were transformed into all strains by electroporation and were maintained during growth by addition of 30 μg ml\(^{-1}\) gentamicin sulfate or 50 μg ml\(^{-1}\) kanamycin sulfate in the media. 5 ml of LB medium (10 g l\(^{-1}\) peptone, 5 g l\(^{-1}\) yeast extract, 10 g l\(^{-1}\) NaCl) in 50 ml polypropylene tubes were used for all liquid cultures, which were incubated for 12 hours at 37°C with shaking at 250 rpm before use. For the generation of mixed colonies, overnight cultures of the relevant strains were
mixed in equal ratios and 2 µl of this mixture were spotted on 1.5% w/v LB agar medium and incubated for 12 hours at 37°C. For experiments involving colony counts as the final read-out, cell suspensions prepared from overnight liquid cultures were diluted appropriately, platted onto 1.5% w/v LB agar medium and incubated for 12 hours at 37°C.

For testing the frequency of plasmid loss in the absence of antibiotics, overnight liquid cultures of *Escherichia coli* MG1655 cells harboring the pUltraGFP-GM plasmid were diluted into 200 µl of LB medium, with and without 30 µg ml⁻¹ gentamicin sulfate, in 96-well plates at a starting optical density A₆₀₀ (OD₆₀₀) of 0.001. Cells were grown for 24 hours at 37°C with shaking at 250 rpm for approximately 15 generations. Subsequently they were analyzed by flow cytometry as described in section 2.5 of the Materials and Methods. Seven biological replicates were analyzed for each tested growth condition (i.e. growth with or without antibiotics).

2.3. Growth rate measurements

Overnight liquid cultures were diluted into 200 µl of LB medium in 96-well plates at a starting OD₆₀₀ of 0.001. Cells were grown with agitation at 37°C using a plate reader (Tecan, Infinite M200 Pro) and their OD₆₀₀ was measured automatically every 900 sec. Growth rates were calculated for individual wells as the slope of the linear regression of the natural logarithm of OD₆₀₀ over time, for OD₆₀₀>0.1 and OD₆₀₀<0.3. For each strain, four biological replicates were analyzed.

2.4. Mammalian cell infection
HeLa cells (ATCC) were maintained in Dulbecco’s Modified Eagle Media (DMEM) (1 g l\(^{-1}\) glucose with addition of 2 mM glutamax and 10% w/v FCS) and routinely passaged every two or three days. For infections, cells were seeded at 7.5x10\(^4\) cells per well into a 24-well plate while a glass coverslip was also inserted in each well. Each bacterial strain harboring either pUtraGFP-GM or pUtraRFP-GM was grown independently and strains were mixed in equal ratios immediately prior to each infection.

Stationary phase cultures of *Shigella sonnei* strain 53G (26) were diluted 1:200 into Tryptone Soy Broth (Oxoid) and incubated for 2 hours at 37°C with shaking at 200 rpm. Bacteria were pelleted and resuspended in DMEM before being added to HeLa cells at a multiplicity of infection (MOI) of approximately 100:1. Infected cells were centrifuged for 10 min at 750 g and then incubated for 30 min at 37°C in the presence of 5% v/v CO\(_2\). Stationary phase cultures of *Salmonella typhimurium* strain SL1344 (27) were diluted 1:33 into LB medium and incubated for 3 hours at 37°C with shaking at 200 rpm. Bacteria were added to HeLa cells to give an MOI of approximately 300:1. Infected cells were incubated for 15 min at 37°C in the presence of 5% v/v CO\(_2\) and then they were washed three times with 1 ml of PBS per well (Dulbecco, pH 7.1-7.5), and further incubated for 60 min. Stationary phase liquid cultures of enterohaemorrhagic *E. coli* (EHEC) strain EDL933 (stx-) (28) were diluted 1:1000 into DMEM and incubated for 16-18 h at 37°C in the presence of 5% v/v CO\(_2\), without shaking. Bacteria were added to HeLa cells to give a MOI of approximately 200:1. Infected cells were centrifuged for 5 min at 500 g and then incubated for 2.5 hours at 37°C in the presence of 5% v/v CO\(_2\).

2.5 Flow Cytometry
1-4 µl of overnight liquid cultures of the relevant strains were diluted into 200 µl of filter-sterilized M9 medium without a carbon source (12.8 g l⁻¹ Na₂HPO₄·7H₂O, 3 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ NaCl, 1 g l⁻¹ NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂). The cell suspensions were analyzed on a BD Accuri C6 Flow Cytometer using 10,000 and 8,000 as thresholds for the FSC and SSC parameters, respectively. 20,000 events per sample were quantified.

2.6 Imaging

For imaging of bacterial cultures at single-cell level, 5 µl of overnight liquid culture were placed onto a square piece of 1.5% w/v LB agar medium and covered with a 1.5 mm-thick cover slip. Epifluorescence images were acquired using a Zeiss Axio Observer inverted microscope with a Zeiss Plan-Apochromat 63x oil immersion objective (NA=1.4), a Zeiss AxioCam MRm camera, a Zeiss Definite Focus system and the associated Zen software. Confocal imaging of liquid cultures was carried out using the Zeiss Plan-Apochromat 63x oil immersion objective on a Zeiss LSM700 scanning laser confocal unit with the associated Zen software. Confocal images of mixed colonies were acquired on the same confocal unit but with a Zeiss Plan-Apochromat 40x objective. In each case, a square piece of 1.5% w/v LB agar medium containing the entire colony was cut out and placed on a slide without a coverslip.

For imaging of mammalian cells, following infections described in section 2.4, all HeLa cells were washed three times with 1 ml of PBS per well (Dulbecco, pH 7.1-7.5) and fixed on the cover slip with 2% para-formaldehyde (PFA) for 20 min at room temperature. PFA was removed, the cells were washed three times with 1 ml of PBS, permeabilized with 0.1% v/v Triton X-100 and their DNA was stained with DAPI (Invitrogen, Thermo Fisher Scientific,
Basingstoke, United Kingdom). Subsequently, the coverslips were washed three times with 1 ml of PBS and mounted on microscope slides using ProLong Gold Antifade mounting media (Life Technologies, Thermo Fisher Scientific, Basingstoke, United Kingdom). Epifluorescence images were acquired on a Zeiss Axio Observer Z1 widefield microscope with a Zeiss LD Plan-Neofluar 40x objective and the associated AxioVision software.

For the quantification of the percentage of *E. coli* MG1655 cells harboring the pUltraGFP-GM plasmid in a mixed culture with cells harboring pUltraRFP-GM, 5 µl of the liquid culture were placed onto a square piece of 1.5% w/v LB agar medium and covered with a 1.5 mm-thick cover slip. Four different fields of view were imaged by epifluorescence microscopy as described above. Subsequently, the images were analysed using a custom ImageJ (29) script. Briefly, the RFP and GFP channels were processed separately with the "Sharpen", "Make Binary" and "Watershed" functions and subsequently, particles with a size above 0.002 were counted. The percentage of green fluorescent cells was calculated as the number of green cells divided by the total number of cells.

3. Results and Discussion

3.1 The pUltra plasmids are versatile and result in bright fluorescent labeling of the entire bacterial population

Plasmids pUltraGFP-KM and pUltraRFP-KM (Table S1) were initially developed in the lab of Eric Klavins (University of Washington) and are synthetic constructs where a GFP or RFP gene is under the control of a strong optimized Biofab promoter (see section 2.1 of the Materials and Methods for more details). The DNA sequence of these original plasmids,
which were constructed by Gibson assembly (30), is given in Fig. S1. pUltraGFP-KM and pUltraRFP-KM were the starting point for generating two series of pUltra plasmids, pUltra-KM (Fig. 1A) and pUltra-GM (Fig. 1B), which confer resistance to kanamycin and gentamicin sulfate, respectively. These constructs do not contain an engineered multiple cloning site; however, it is possible to use their EcoRI and BamHI restriction sites (Fig. 1) to replace the fluorescent protein gene as long as the Biofab promoter sequence is included in the forward oligonucleotide primer which is used to amplify the fluorescent protein gene of choice. This is the strategy we followed to generate pUltraYFP-KM and pUltraCFP-KM (Table S1). Subsequently, we produced the pUltra-GM series by inserting a gentamicin resistance cassette in the NruI site (Fig. 1) which is located in the kanamycin resistance cassette of the pUltra-KM plasmids, and hence disrupting this cassette. The same strategy can be used to generate pUltra plasmids conferring any antibiotic resistance of choice.

The pUltra plasmids have a p15A origin which replicates successfully in most Enterobacteria (31) as well as some other Gammaproteobacteria like Shewanella (20) and Haemophilus (22) species, while the Biofab promoter, containing conserved -10 and -35 regions, should be broadly active in Gram-negative bacteria. Although these plasmids have a medium to low copy number (14-16 copies per cell (32)), transformation of both pUltra-KM and pUltra-GM constructs into E. coli strain MG1655 (Table S3) results in the production of vast amounts of fluorescent protein that makes individual cells visible, even in colonies growing on solid medium. All characterization experiments presented in this study were performed with the pUltra-GM constructs but we got the same results when we used the pUltra-KM plasmids.

Overnight liquid cultures of E. coli MG1655 cells containing the four variants of the pUltra-GM plasmids (expressing GFP, RFP, YFP or CFP, Table S1) were imaged using
epifluorescence (Fig. 2A) and confocal (Fig. 2B) microscopy. Despite their medium to low copy number (32) and the use of minimal exposure time (50 ms for GFP; 200 ms for RFP; 250 ms for YFP; 230 ms for CFP) or laser power during epifluorescence or confocal imaging, respectively, all plasmids provided very bright labeling of bacterial cells and could be used with the same success for both microscopy techniques. In addition, according to the acquired epifluorescence images (Fig. 2A), the entire population was labeled in a uniform way. No fluorescent signal was detected when the same microscopy settings were applied to the non-recombinant E. coli MG1655 strain (Fig. S2).

3.2 The pUltra plasmids allow unambiguous distinction of differently colored cells

We assessed how the pUltra-GM plasmids performed when bacterial cells labeled with different colors were imaged simultaneously and compared them with another set of constructs for fluorescent labeling. We chose a series of mini-Tn7-based constructs which will be referred to as pTn7 plasmids from now on (Table S1). When these constructs are used in conjunction with a helper plasmid encoding the Tn7 site-specific transposition pathway, they can integrate a fluorescent protein gene in the chromosome of a broad range of bacterial species (21) but when they are transformed into E. coli without the helper plasmid they result in production of fluorescent protein expressed from the plasmid. We chose the pTn7 constructs for comparison since they are available in the same selection of colors as the pUltra-GM plasmids and they, like the pUltra-GM plasmids, confer resistance to gentamicin sulfate.

Growth rate measurements of E. coli strain MG1655 transformed with all eight plasmids (pUltra-GM and pTn7) were carried out in LB medium to assess the fitness cost associated
with each of these constructs (Fig. 3). The average doubling time of all the tested strains in exponential phase was similar and did not differ significantly from the control E. coli strain MG1655. This means that there are no detectable growth rate effects in pure culture for any of the pUltra-GM or pTn7 plasmids in LB medium. However, for strains harboring the pTn7 constructs, we observed a significant initial lag phase in the recorded growth curves (data not shown), indicating that although in exponential phase these plasmids do not burden the bacteria it is possible that they do cause some stress during late exponential and stationary growth phase. This could be due to the copy number of the pTn7 plasmids (45-60 copies per cell (33)) which is higher than the medium to low copy number (14-16 copies per cell (32)) of the pUltra-GM constructs.

Mixed liquid cultures of several color combinations (green-red, yellow-red and in some cases blue-red) for both strains were imaged by epifluorescence (Fig. 4A) and confocal microscopy (Fig. 4B). Since all acquired images were optimized individually, the examples shown in Fig. 4 represent the best possible results that can be achieved by using each plasmid series. For pUltra-GM plasmids all bacterial cells were labeled uniformly and differently colored cells could be unambiguously distinguished without any crosstalk between the tested fluorophores. This was not the case for the pTn7 plasmids; with the exception of the RFP/CFP-labeled mixture (Fig. 4A), in most cases only a very small part of the bacterial population displayed any fluorescence and in the case of confocal imaging there was crosstalk between the fluorescence signals that were recorded (for example in the top right panel of Fig. 4B several cells appear yellow as a result of green fluorescence being detected on the red channel). In addition significantly higher exposure times or laser power settings were used for the strains transformed with the pTn7 constructs compared to strains harboring the pUltra-GM constructs (an example of greater exposure times during epifluorescence microscopy being
RFP; 3000 ms and CFP; 250 ms for the pTn7 plasmids compared to RFP; 200 ms and CFP; 150 ms for the pUltra-GM plasmids). The same result was obtained when mixed colonies of GFP/RFP- or YFP/RFP-labeled bacteria were imaged by confocal microscopy (Fig. 4C). Unlike the pTn7 plasmids, when the pUltra-GM plasmids were used, the entire bacterial population was efficiently labeled and the fine structures which arise when bacteria grow on solid medium were clearly visible without any crosstalk between the fluorophores.

We tested if the pUltra-GM plasmids would give satisfactory results in distinguishing fluorescent populations of different colors by flow cytometry (Fig. 5). Mixtures of approximately equal ratios of GFP/RFP- or YFP/RFP-labeled cells were analyzed and while for pUltra-GM labeling, the fluorescent signals were well-separated and could be distinguished unambiguously, the distinction of the different colors was not possible for pTn7-labeled cells. In addition, the GFP or YFP signals for the pUltra-GM mixtures (Fig. 5, top and bottom left) are positioned at significantly higher values than in the case of the pTn7-labeled cells (Fig. 5, top and bottom right) ($10^5$ (arbitrary fluorescent units) for GFP and $10^4$ for YFP when the pUltra-GM plasmids were used compared to less than $10^4$ for GFP and $10^3$ for YFP when the Tn7 plasmids were used). This confirms that the amount of fluorescent protein produced by the pUltra constructs is much greater than that produced by the Tn7 plasmids and explains the greater discrimination power of the former.

We also wanted to compare the efficiency of distinction of differentially-labeled fluorescent cells by microscopy and flow cytometry when using the pUltra-GM plasmids to a more traditional method, like colony counts. We prepared a mixture of almost equal ratios of GFP/RFP-labeled cells and subsequently we quantified the percentage of GFP-labeled cells by epifluorescence microscopy, flow cytometry and colony counts (Fig. S3). The results we
obtained by both methods which rely on the detection of a fluorescent signal (flow cytometry and epifluorescent microscopy) were comparable to the result obtained by colony counts. This means that the fluorescence signal provided by the pUltra constructs is high enough to allow their use for accurate quantification of differentially-labeled bacterial populations.

3.3 The pUltra plasmids can be used for studying bacterial pathogens and their interaction with their hosts

We transformed S. sonnei, S. typhimurium and EHEC strains (Table S3) with pUltraGFP-GM and pUltraRFP-GM and we used approximately equal ratios of differently colored bacteria of each species to infect HeLa cells. After the infection process (section 2.4 of the Materials and Methods), the mammalian cells were extensively washed to remove any non-internalized bacteria, fixed to microscope slides and imaged by epifluorescence microscopy (section 2.6 of the Material and Methods) (Fig. 6). From the acquired images, it was obvious that for all pathogenic species, bacteria were clearly visible inside or adhered to the surface of the host cell and their color (red or green) could be unambiguously identified. In addition, the fixation process which is necessary for mammalian cell visualization did not affect any of the tested fluorophores. This means, that the pUltra-GM plasmids are highly appropriate for tracking pathogenic enteric bacteria during their interaction with mammalian cells.

Finally, we assessed the stability of the pUltra plasmids in vitro to establish if they can be used in studies where the use of antibiotics would be limited. We grew E. coli MG1655 cells harboring pUltraGFP-GM with and without gentamicin sulfate in LB medium for 15 generations (approximately 24 hours) and subsequently quantified the percentage of cells not displaying any detectable fluorescence by flow cytometry (Fig. S4). We found that in the
absence of gentamicin sulfate, 6% of the bacterial population was unlabeled after 24 hours of growth whereas when gentamicin was added to the medium, only 1.5% of cells did not show any detectable fluorescence. Despite the fact that the loss of the plasmid almost triples in the absence of antibiotics in vitro, the percentage of remaining cells that display a strong fluorescent signal is still very high (approximately 94%). While there are numerous tools and methodologies for fluorescent labeling of bacteria, and especially pathogens, during animal experiments (for example (23,24)) and while the pUltra plasmids were not developed specifically for in vivo labeling, their in vitro stability is promising enough in order for these constructs to be considered for experiments requiring in vivo labeling.

3.4 Conclusion

We have described the construction and characterization of the pUltra plasmids, a series of synthetically optimized constructs which replicate efficiently in Enterobacteria and several Gammaproteobacteria. The pUltra plasmids are equipped with a strong Biofab promoter (25) which ensures constant and high level of transcription of the fluorescent protein gene. This results in bright labeling of the entire bacterial population, which can be imaged equally successfully by epifluorescent or confocal microscopy. The medium to low copy number of the plasmids (14-16 copies per cell (32)) does not interfere with the physiology of the cells while the abundant production of the fluorophore overcomes the shortfalls of low signal intensity. The latter are normally dealt with by using higher exposure times or higher laser power settings and can lead to crosstalk between fluorescent proteins. In addition, by using the pUltra plasmids one can unambiguously distinguish bacteria labeled with different colors both by microscopy and flow cytometry and, in the case of some pathogenic Enterobacteria, even while the pathogens are inside a mammalian cell. Therefore, we propose that the pUltra
plasmids are overall excellent tools for the fluorescent labeling of bacterial populations and more specifically that they have the potential to facilitate the study of ecosystems composed of different bacterial species or of the interactions of enteric pathogens with their host organisms.

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Figure legends

**Figure 1.** Vector map of the A) pUltra-KM and B) pUltra-GM plasmid series. The origin of replication (p15A), the antibiotic resistance gene (Kan\(^R\) or Gent\(^R\)), the strong optimized synthetic promoter (Biofab), the fluorescent protein gene (sfGFP, mRFP1, eYFP and eCFP) and the terminator (his operon) are indicated. Depending on the construct, these plasmids constitutively express superfolder GFP (sfGFP), monomeric RFP (mRFP1), enhanced YFP (eYFP) or enhanced CFP (eCFP) and can be maintained in cultures using kanamycin (A) or gentamicin sulfate (B). These plasmids do not have a multiple cloning site, however the fluorescent protein gene can be replaced using the EcoRI and BamHI restriction sites, and the antibiotic resistance of the plasmid can be changed using the NruI restriction site(s) (see section 2.1 of the Materials and methods).

**Figure 2.** A) Epifluorescence and B) confocal microscopy on liquid cultures of *Escherichia coli* strain MG1655 transformed with the pUltra-GM plasmid series. Images were acquired as described in section 2.6 of the Materials and Methods, three replicates were performed and characteristic images are shown here. All four fluorescence plasmids lead to efficient labeling of bacterial cells, which can be visualized equally well with both microscopy techniques. The epifluorescent images (A) show that in each case the entire bacterial population is labeled. The same liquid cultures were used for parts (A) and (B), but different fields of view are displayed. Scale bars are 5 \(\mu\)m.

**Figure 3.** Comparison of the effect on growth rate of the pUltra-GM and the pTn7 plasmid series. The average doubling time in exponential phase does not significantly differ between strains harboring pUltra-GM plasmids, pTn7 plasmids and the control MG1655 *Escherichia*
coli strain, and hence, there is no major fitness cost associated with any of the tested plasmids. Growth rate measurements were conducted as described in section 2.3 of the Materials and Methods and the error bars represent the standard deviation of four biological replicates.

**Figure 4.** A) Epifluorescence microscopy on mixed liquid cultures, B) confocal microscopy on mixed liquid cultures and C) confocal microscopy on mixed colonies of *Escherichia coli* strain MG1655 transformed with the pUltra-GM plasmid series (left) or the pTn7 plasmid series (right). For the preparation of mixed liquid cultures, each bacterial strain was grown independently and subsequently, strains were mixed in equal ratios. Mixed colonies were generated as described in section 2.2 of the Materials and Methods. Images were acquired as described in section 2.6 of the Materials and Methods, three replicates were performed and characteristic images are shown here. The same liquid cultures were used for parts (A) and (B) but different fields of view are displayed. The acquisition of each image was optimized independently, hence they represent the best possible result that can be achieved by using each plasmid series. In every experiment, the pUltra-GM plasmids allow efficient labeling of the entire bacterial population and unambiguous distinction between GFP- and RFP-, YFP- and RFP-, and CFP- and RFP-labeled cells at single-cell level as well as in structured growth environments like colonies. The same cannot be done by using the pTn7 plasmids. Scale bars are 5 μm for parts (A) and (B) and 20 μm for part (C).

**Figure 5.** GFP- and RFP-labeled, as well as YFP- and RFP-labeled, cells can be distinguished unambiguously using a basic flow cytometer when the pUltra-GM plasmids are used (left) but not when the pTn7 plasmids are used (right). Fluorescence was recorded in the green channel. Flow cytometry was carried out as described in section 2.5 of the Materials
and Methods, four replicates of each experiment were performed and a representative example is shown here.

**Figure 6.** Epifluorescence microscopy on HeLa cells infected with equal ratios of GFP- and RFP- labeled A) *Shigella sonnei*, B) *Salmonella typhimurium* and C) enterohaemorrhagic *Escherichia coli*. The pUltraGFP-GM and pUltraRFP-GM plasmids were used. Mammalian cell infection and imaging were carried out as described in sections 2.4 and 2.6 of the Materials and Methods, respectively. Three replicates of each experiment were performed and characteristic images are shown here. *S. sonnei* (A) and *S. typhimurium* (B) are visible inside the infected mammalian cell while enterohaemorrhagic *E. coli* can be seen adhered to the HeLa cell surface (C). GFP- and RFP-expressing bacteria can be clearly distinguished in all infections. The nucleus of the HeLa cell is stained blue with DAPI and the contour of the cell can be observed by phase contrast. Scale bars are 10 µm.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
HIGHLIGHTS:

- The pUltra plasmids are designed for fluorescent labeling of Enterobacteria.
- pUltra vectors with two antibiotic resistances are available in four colors each.
- Labeling is efficient and uniform without any growth rate effects to the cells.
- Color distinction during microscopy and flow cytometry is entirely unambiguous.
- The plasmids can label enteric pathogens even in association with their host cells.