

# **BBF RFC 59: Quantitative measurement of mammalian cell invasion by bacteria using flow cytometry**

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## **1. Purpose**

Purpose of this RFC is to provide method of measurement of bacterial invasion into mammalian cells. Also this RFC defines Percent of INvasion (PIN) unit.

## **2. Relation to other BBF RFCs**

This RFC is not related to any other Request For Comments document.

## **3. Copyright Notice**

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## **4. Prerequisites for invasion measurement**

Each invasive bacterial strain that is to be measured **MUST** express GFPmut3b (eg. BioBrick part BBa\_E0040) as reporter protein. The reporter protein **SHOULD** be coexpressed in single operon with invasion-inducing proteins. BioBrick part BBa\_E0840 **SHOULD** be used for this purpose for the experiments conducted using E. coli strains.

Measurement **MUST** be done using flow cytometer capable of reading fluorescence at 510 nm and equipped with 488 nm excitation laser.

Following control bacterial strains **MUST** be included in the experimental setup:

- noninvasive bacterial strain that do not express any fluorescent protein
- noninvasive bacterial strain that constitutively expresses high levels of GFPmut3b (for E. coli measurements BioBrick BBa\_K299817 SHOULD be used for this purpose)

Invasive bacterial strain expressing GFPmut3b SHOULD be used as a positive control wherever possible (in experiments with E. coli strains BioBrick BBa\_K299816 SHOULD be used for this purpose). All bacterial strains used in the experiments SHOULD be resistant to ampicillin and streptomycin because mixture those antibiotics is very common supplement in mammalian cell culture media. Additionally all used strains MUST be sensitive to Kanamycin and Gentamycin. This allows efficient killing of the bacteria during sample preparations and serves as additional safety measure during work with the strains.

## **5. Mammalian cell cultures**

The cells SHOULD be grown in a vessel that holds minimum 100 000 cells at 85% confluence. 6 well plates are recommended for this purpose. After unfreezing from stock the cells MUST be passaged at least twice before measurement can be made. Penicillin-streptomycin mixture SHOULD be added to the culture media to ensure that it's free from bacterial contamination. Measurement SHOULD be made when the cells are in 85-90% confluence and there are 50 000 – 80 000 cells in each sample.

## **5. Bacterial cell cultures**

For measurement purposes overnight bacterial cultures SHOULD be used to obtain maximum culture density. Culture media SHOULD be supplemented with 100 ug/ml ampicillin and 50 ug/ml streptomycin in order to induce expression of resistance genes for these antibiotics. In order to minimize the volume of bacterial suspension added to the mammalian cells bacterial cultures MUST be condensed before measurement by centrifugation and resuspension in sterile PBS. The amount of bacterial suspension added to the mammalian cell culture MUST NOT exceed 1/20 of the culture volume.

## **6. Sample preparation protocol**

The day before measurement mammalian cell cultures MUST be feed with the fresh medium.

Sample preparation begins with replacing mammalian cell culture medium with fresh medium without serum. After that the cells MUST be incubated for 30 minutes and appropriate amounts of bacterial cell suspensions MUST be added. After incubation of mammalian cells with the bacteria cell culture vessels MUST be washed twice with PBS supplemented with 100ug/ml kanamycin and trypsinized for 10 minutes at 37 C. Trypsin MUST be inactivated by addition of equal volume of culture medium containing 10% serum. Then the cells MUST be centrifuged for 10 minutes at 800 RPM and supernatants MUST be removed.

The cells must be resuspended in culture medium supplemented with 100 ug/ml lysozyme and 100 ug/ml kanamycin. The suspension must be incubated at 37 C for 10 minutes to allow lysozyme to digest bacteria that are outside mammalian cells. The time between end of the incubation and measurement of the sample on the flow cytometer SHOULD NOT exceed 10 minutes.

## **7. Flow cytometer setup and definition of the PIN units**

The fluorescence acquisition filter MUST be set at 510 nm and the excitation filter must be set at 488 nm. The control strains MUST be used to adjust the gates and counting markers in the following manner:

- cells infected with noninvasive, nonfluorescent bacteria MUST be captured in acquisition gate discriminating events by forward scatter and side scatter
- bacteria expressing GFP MUST NOT be captured in acquisition gate discriminating events by forward scatter and side scatter
- cells infected with noninvasive, nonfluorescent bacteria and with bacteria expressing GFP MUST be within 'noninvaded cells' counting marker on fluorescence histogram
- population of the cells infected with invasive control strain showing significantly higher fluorescence than those in 'noninvaded cells' counting marker MUST be

included in 'invaded cells' counting marker

The acquired data MUST be expressed in PIN (Percent of Invasion) units which is the percent of all of gated events that fall within 'invaded cells' counting marker. Additionally percent of all of gated events that fall within 'noninvaded cells' counting marker SHOULD be presented with the data.

## **8. Author's Contact Information**

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