Flow CAST®

Basophil Activation Test (BAT)
Flow Cytometry

This product is for research use only
It is not intended for use in diagnostic procedures

FK-CCR-U 100 tests

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ENGLISH

INTENDED USE

The Flow CAST® kit is a basophil activation test (BAT) and is intended for the determination of expression of CD63 surface marker on basophils in whole blood by flow cytometry upon antigen stimulation. This product is for research use only. It is not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The assay is based on the method first described by Sainte-Laudy *et al.* 1994 and 1996 (1,2) where basophil activation by allergens or controls is detected by flow cytometry measured by the increase of the CD63 (gp53) at the cellular surface. IgE and non-IgE mediated reactions can be detected (3-5).

Stimulation Buffer and Allergen is added to EDTA whole blood from individuals. The allergen mimics the *in vivo* reaction where specific IgE bound to the cellular surface are bridged by the culprit allergen and activates an intracellular signaling cascade leading to the activation of the basophil. As a consequence, intracellular compounds bearing the transmembrane protein CD63 are fused to the cellular membrane and therefore exposed to the extracellular matrix.

As positive control, highly specific monoclonal antibody binding to the high affinity IgE binding receptor (FcεRI) or the unspecific cell activator fMLP is used.

Together with the cellular stimulation, Staining Reagent is added containing a mixture of monoclonal antibodies to human CD63 labeled with fluorescein isothiocyanate (anti-CD63-FITC) and to human chemokine receptor CCR3 labeled with phycoerythrin (anti-CCR3-PE). CCR3 is constitutively expressed on eosinophils and basophils (6,7)

Erythrocytes are removed by a lysing reaction and after a short centrifugation step the cells are resuspended in Wash Buffer and analyzed by flow cytometry (*cf.* Flow cytometric Data Acquisition on page 4).

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Stimulation Buffer containing calcium, heparin and IL-3	1 vial lyoph.	B-CCR-STB	Reconstitute with 50 ml of water [1]
Stimulation Control anti-FcεRI mAb	1 vial lyoph.	B-CCR-STCON	Reconstitute with 1.5 ml of B-CCR-STB
Stimulation Control fMLP[2]	1 vial lyoph.	B-CCR-FMLP	Reconstitute with 1.5ml of B-CCR-STB
Staining Reagent Mix of anti-CD63-FITC and anti-CCR3-PE mAb	1 vial 2.2 ml	B-CCR-SR	Ready to use
Lysing Reagent [3] 10x concentrated	1 vial 25 ml	B-CCR-LYR	Dilute with 225 ml of deionized water
Wash Buffer	1 vial 100 ml	B-CCR-WB	Ready to use

Table 1

[1] For required water quality, see Chapter Procedural Notes
[2] N-formyl-methionyl-leucyl-phenylalanine
[3] Crystals may be formed during storage at 2-8°C and should be dissolved at 18-28°C prior to dilution.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened reagents	
Store at 2-8°C. Do not use past kit expiration date.	
Opened / reconstituted reagents	
Stimulation Buffer	Stable at –20°C for 6 months. Aliquot if repeated use is expected.
Stimulation Control	
Stimulation Control fMLP	Stable at –20°C for 6 months. Aliquot if repeated use is expected.
Lysing Reagent	Stable at 2-8°C for 6 months.
Staining Reagent	Stable at 2-8°C until expiration date.
Wash Buffer	

Table 2

ALLERGENS SUPPLIED UPON REQUEST

Order codes: see BÜHLMANN Allergen list on the webpage (www.buhlmannlabs.ch)

- **Protein Allergens:** The BÜHLMANN protein allergens are quality controlled and shipped in liquid, concentrated form (1µl/vial). The protein allergens need to be stored refrigerated and must be diluted before use.
- **Drug and Chemical Allergens:** The BÜHLMANN low molecular weight allergens are shipped in lyophilized form. The low molecular weight allergens need to be stored refrigerated and must be reconstituted before use.

Refer to the BÜHLMANN Allergen Booklet and **Allergen Data Sheets** available on the BÜHLMANN webpage (www.buhlmannlabs.ch/.

ALLERGEN REAGENTS FROM OTHER SOURCES

Allergens from other sources might be used in the Flow CAST® assay with the following limitations:

- No matrix-bound allergens (solid or liquid phase).
- No allergen preparations containing cytotoxic compounds (stabilizers, preservatives) such as glycerol, phenol, sodium azide or merthiolate (thimerosal).

For the procedure to establish customer specific allergens for the CAST®-Assays ask your local distributor or contact BÜHLMANN.

WARNINGS AND PRECAUTIONS

Reagents Containing Human Source Material:

No kit components contain material of human origin.

All samples should be handled as if capable of transmitting infections and reasonable precautions should be taken.

MATERIALS REQUIRED BUT NOT PROVIDED

- K-EDTA venipuncture tubes.
- Centrifuge for centrifugation at 500 x g.
- Disposable, pyrogen-free polypropylene or polystyrene test tubes and appropriate test tube racks for the stimulation
  NOTE: Polystyrene tubes should fit with the Flow Cytometer used (*e.g.* 12 x 75 mm FALCON tubes from Becton Dickinson; order code: 352052).
- Vortex Mixer.
- Precision pipettes with disposable, pyrogen-free tips:

10-100 µl, 100-1000 µl, 1-5 ml adjustable pipette and a 10-50 µl adjustable dispenser.
- Cylinder for preparing the Stimulation Buffer.
- Sterile, ultrapure and apyrogenic water for preparing the cell stimulation reagents (*cf.* Chapter Procedural Notes).
- Water bath set at 37°C.
- Distilled or deionized water as well as beaker or cylinder for the preparation of Lysing Reagent.
- Bottle-top dispensers for Lysing Reagent and Wash Buffer, respectively.
- Flow Cytometer with 488 nm (blue) excitation wavelength including appropriate software (cf. chapter Flow cytometric Data Acquisition).

SPECIMEN COLLECTION AND STORAGE

It is recommended that s should avoid systemically administered antiallergenic drugs such as corticosteroids, chromoglycic acid (DSCG) for at least 24 hours prior to blood sampling.

Collect sufficient blood into **K-EDTA venipuncture tubes**. Fill the venipuncture tubes up to the dedicated volume with blood. Not sufficiently filled tubes (filling grade < 50%) lead to higher EDTA concentration in the sample and thus may give false negative results.
1 ml of whole blood is sufficient for about 18 test tubes.
Perform the cell stimulation immediately or store the blood sample refrigerated (2-8°C) for up to 48 hours**.** For detecting responses to drugs store the blood sample only up to 24 hours. **Do not centrifuge or freeze blood samples**.

PROCEDURAL NOTES

- RECOMMENDED WATER QUALITY FOR THE FLOW CAST®. The use of sterile, ultrapure and apyrogenic water for reconstituting Stimulation Buffer (B-CCR-STB) is essential for good and reproducible basophil stimulation. The following sources of water may be used: Cell culture grade water, infusion grade water or deionized, double distilled water that is ultra filtrated in a periodically sanitized 10 kDa ultra filter.

  The Lysing Reagent (B-CCR-LYR) can be reconstituted with deionized, double distilled water or the same water quality that is used for the cell stimulation reagents.
- PRECAUTIONS TO AVOID ALLERGEN CONTAMINATION DURING CELL STIMULATION. Aeroallergens in the laboratory may contaminate open blood samples. Therefore, care must be taken to cover blood samples and cell stimulation tubes. Avoid dust mites, pollinating plants, latex gloves or equipment potentially containing latex and open windows in the laboratory where the cell stimulation is performed. Therefore, we recommend carrying out the cell preparation and stimulation steps in a laminar flow hood.
- For cell stimulation and labeling reaction, the use of tissue culture grade MICROTITER PLATES is possible.

ASSAY PROCEDURE

Important: The following procedure is optimized for whole blood specimen collected with EDTA as anticoagulant.

1. Mix the anti-coagulated blood sample by inverting the venipuncture tube several times.
2. Prepare fresh and pyrogen-free 3.5 ml polypropylene or polystyrene tubes suitable for Flow Cytometry measurements.
3. For each sample, label the tubes *e.g.*:
   PB = sample background
   PC1 = stimulation control with anti-FcεRI Ab
   PC2 = stimulation control with fMLP
   A1-1 for allergen 1 with dilution 1
   A1-2 for allergen 1 with dilution 2
   etc.

Stimulation and Staining

4. Pipet 50 µl of the corresponding stimulus to each tube for each sample
   PB tube: 50 µl of **Stimulation Buffer (background)**
   PC1 tube: 50 µl of **Stimulation Control**
   PC2 tube: 50 µl of **Stimulation Control fMLP**
   Ax-y tube: 50 µl of **Allergen**
5. Add 100 µl of Stimulation Buffer to each tube.
6. Add 50 µl of sample`s whole blood to each tube. Be sure that the side wall and top of the tube are free of blood.
7. Mix gently.
8. Add 20 µl Staining Reagent to each tube.
9. Mix gently, cover the tubes and incubate for 15 minutes at 37°C in a **water bath**.
   (using an incubator will take about 10 minutes longer incubation time due to less efficient heat transfer).

Lysing

10. Add 2 ml pre-warmed (18-28°C) Lysing Reagent to each tube, mix gently.
11. Incubate for 5 -10 minutes at 18-28°C.
12. Centrifuge the tubes for 5 minutes at 500 x g.
13. Decant the supernatant by using blotting paper.
14. Resuspend the cell pellet with 300 µl of Wash Buffer.

Note: Depending on Flow cytometry instrumentation more wash buffer (e.g. 800 µl) might be necessary.

15. Vortex gently.
16. Acquire the data on the flow cytometer within the same day. If the samples are stored for several hours it should be kept protected from light at 2-8°C.

Note: Samples stored, protected from light at 2-8°C over night are still analysable. A slight decrease of fluorescence intensity and a lower basophil recovery can be observed.

FLOW CYTOMETRIC DATA ACQUISITION

Flow cytometric acquisition can be performed on any flow cytometer working with a 488 nm argon laser diode (blue-green excitation light).
The flow cytometer must be equipped to detect Forward Scatter (FSC), Side Scatter (SSC) and the two fluoro-chromes FITC and PE.
Ensure that the flow cytometer is properly aligned and colour compensation is set.
During acquisition of the samples, make sure that on a FSC/SSC histogram the leukocyte population is separated into three discrete populations. Adjust the amplification (gain) of FSC and SSC signals to obtain a distribution that is shown in Figure 1. Refer to the flow cytometer product manuals for instructions.

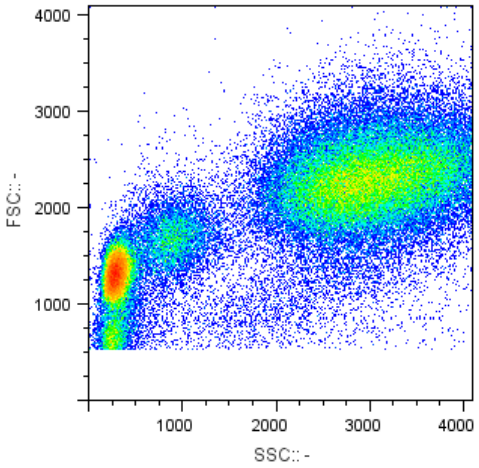


Figure 1: Three discrete populations (lymphocytes, monocytes and granulocytes) in FSC/SSC histogram.

Typically, after 500-600 basophilic cells (gated as shown in figure 2) the acquisition can be stopped. At least 200 basophilic cells must be analyzed, requiring a total amount of 50'000-100'000 leukocytes to be acquired per sample. Because of the lower activation percentage in drug allergies each laboratory has to define its own confidence limits (i.e. in drug allergies the limit of basophilic cells analyzed should be set to 300 or more).

DATA ANALYSIS

The analysis of the acquired data can be performed with any flow cytometry analysis software e.g. FlowJo, FloMax, CellQuest or others.
The analysis is based on two steps:

1. Set a gate 1 (R1) by including the entire basophil population CCR3 ^{pos} with low Side Scatter SSC ^{low} (see Figure 2). eosinophils located on the high right side will be excluded due to their SSC ^{high} position.

2. Calculate the percentage of CD63 positive cells (brightly fluorescent FITC; Q2) compared to the total amount of basophilic cells gated in R1 (see Figure 3 and Figure 4).

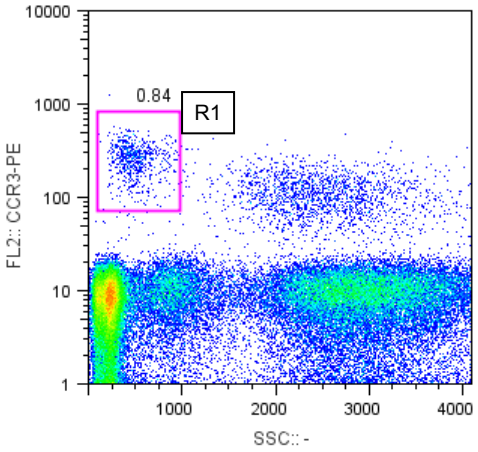
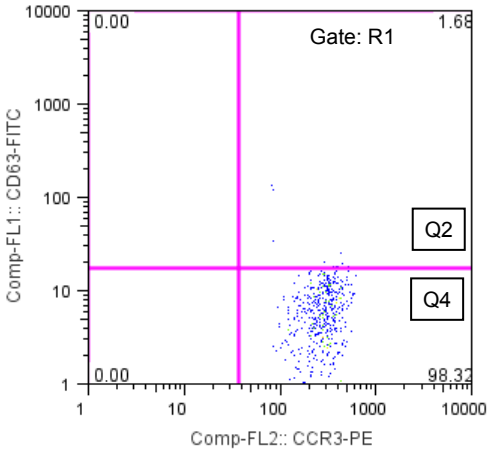
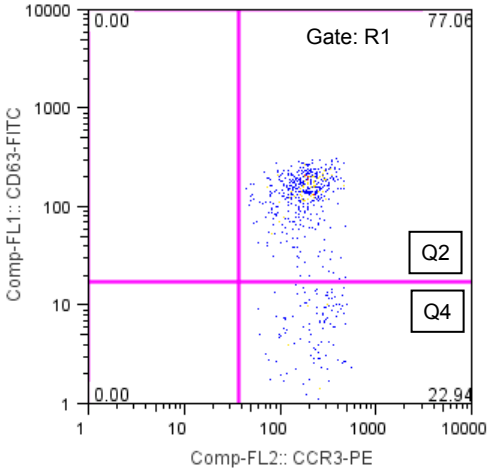


Figure 2: Selection of basophilic cells CCR3 ^{pos} / SSC ^{low}



Gated Region	Count (n=)	%
Total	78251	100.0
R1	655	0.8
Q2 (CD63 ^{pos})	11	1.7
Q4 (CD63 ^{neg})	644	98.3

Figure 3: Sample Background (PB) with STB only



Gated Region	Count (n=)	%
Total	72916	100.0
R1	606	0.8
Q2 (CD63 ^{pos})	467	77.1
Q4 (CD63 ^{neg})	139	22.9

Figure 4: Stimulation Control (STCON)

LIMITATIONS

- Flow cytometry may produce false results if the cytometer has not been aligned perfectly, if the fluorescence emission has not been correctly compensated and if the regions have not been carefully positioned.
- Verify the preparations by eye to assess the efficacy of lysis. The erythrocytes may be incompletely lysed and appear on a light diffraction histogram in the same location as the leucocytes.

QUALITY CONTROL

For the appropriate evaluation of results, different values should be taken into account:

a) The appearance of the three **typical leukocyte populations** (lymphocytes, monocytes and granulocytes) in the FSC/SSC plot can be regarded as a criterion for the quality of the blood sample (time of collection, storage).

b) The **absolute number of basophils** recovered and evaluated, indicates wether the test has been properly performed and a sufficient number of basophils has been counted in order to achieving a statistically relevant difference from the controls. To our experience, the number of basophils to be analyzed should not be below 200 cells.

c) The percentage of activated basophils. In the **negative control** (background) is usually below 5%. Sometimes, however, the percentage of activated basophils in the negative control might be much higher (see **Figure 5**). This may be due to some in vivo basophil activation which indicates recent allergen exposure (*e.g.* pollen allergic people during the pollen season, blood sampling following food or drug allergen exposure). Occasionally, it may also be due to some technical mishap such as contact of the basophils *in vitro* with inappropriate plastic or reagent resulting in non-specific basophil activation. To interpret sample results as positive the allergen specific basophil activation must show at least a Stimulation Index (SI) of 2 (see next chapter).

d) **Positive control** (stimulation control). Two different positive controls are included into the kit. **Anti-FcεRI mAb** mimics the bridging of the receptor caused by the allergen binding to two bound Immunoglobulin E. **fMLP** is a tripeptide causing basophil activation in a non-immunologic way. If one of those two controls shows activation of **>10%** basophils the sample can be regarded as evaluable. Internal evaluation showed 6.1% (n=98) from normal blood donors were negative (non-responder) stimulated with anti-FcεRI and 4.9% (n=61) were negative with fMLP. No sample was negative for both positive controls.

ASSAY PERFORMANCE

Specificity: The anti-CCR3 mAb is a highly specific antibody described in 6 and 7. CCR3 is constitutively expressed on eosinophilic and basophilic leukocytes (see Fig. 2) and in a smaller part on $CD3^+$ cells (lymphocytes). Samples from eight normal blood donors were double stained twice with anti-CCR3-PE and anti-CD3-AF647. The relative amount (mean) of $CD3^+$ cells within the gated Basophil population was 3.9% (cf .**Table 3**)

Basophil Recovery: >500 basophils/stimulation tube. We analyzed 102 samples from normal blood donors and allergic individuals. The median Basophil recovery was 526 cells (95% CI 481-578 basophils) stained with CCR3-PE

Precision (Individual's background): 16.2%CV. The precision shows the reproducibility of the individual's background within the same blood sample incubated 20 times with Stimulation Buffer and consecutively analyzed by flow cytometry. The results are expressed in Table 4 as percentage basophils activated and as mean fluorescence intensity (MFI) of CD63-FITC.

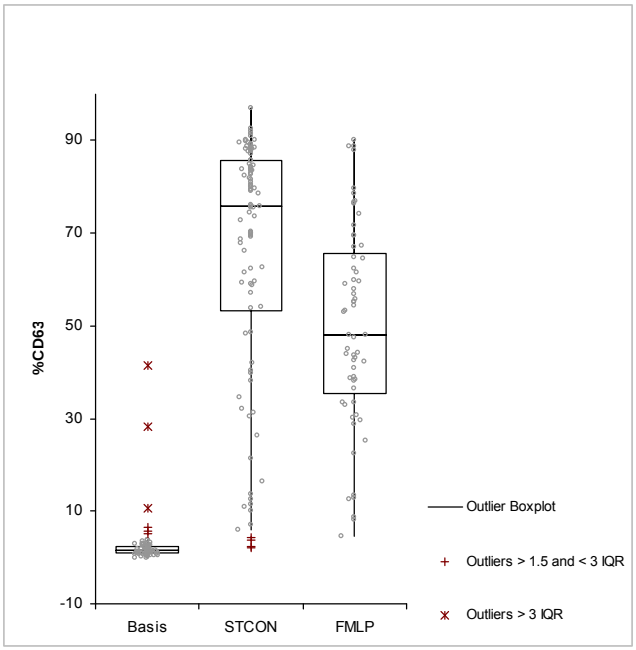
Precision (Positive control): 5.4%CV. The precision shows the reproducibility of the stimulation within the same blood sample incubated 20 times with positive control (STCON) and consecutively analyzed by flow cytometry. The results are expressed in Table 4 as percentage basophils activated and as mean fluorescence intensity (MFI) of CD63-FITC.

Inter-Technician Variation (Positive control): 3.7-8.1%CV. Two blood samples were tested with the Flow CAST® by five different technicians within the same day. The two positive controls included into the kit, STCON and FMLP were used in duplicates.
The results are expressed in Table 5 as mean percentage basophils activated ($CD63^+$) from double activation per technician.

Table 3 Specificity

n	16
Mean	3.85%
95% CI	2.52 – 5-18%
SD	2.50%
Median	3.10%
97.9% CI	1.70 – 5.54%

Figure 5: Box Plot



Positive and negative controls from normal blood donors. Basis: Negative control (n=98); STCON: positive control anti-FcεRI mAb (n=98); FMLP: fMLP positive control (n=61)

Table 4 Precision

	Sample Background		STCON	
	%CD63$^+$	MFI CD63$^+$	%CD63$^+$	MFI CD63$^+$
Mean	2.4%	10.1	35.5%	82.1
SD	0.4%	0.8	1.9%	7.2
%CV	**16.2%**	**7.7%**	**5.4%**	**8.8%**

Table 5 Inter Technician Variation

	S1 (%CD63$^+$)		S2 (%CD63$^+$)	
	STCON	FMLP	STCON	FMLP
Mean	64.8	42.2	69.6	48.1
SD	3.6	1.6	2.6	3.9
%CV	**5.6%**	**3.7%**	**3.7%**	**8.1%**

APPENDIX II
REFERENCES

1. Sainte-Laudy, J, et al. [Analysis of membrane expression of the CD63 human basophil activation marker. Applications to allergologic diagnosis]. Allerg Immunol (Paris) **26**, 211-4. (1994).
2. Sabbah, A and Sainte-Laudy, J. Flow Cytometry applied to the analysis of Lymphocyte and Basophil activation. ACI International **8**, 116-9 (1996).
3. Sanz, ML, et al. Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics. Clin Exp Allergy **32**, 277-86. (2002).
4. DeWeck, AL and Sanz, ML. Flow cytometric cellular allergen stimulation Test (FAST/Flow-CAST): technical and clinical evaluation of a new diagnostic test in allergy and pseudo-allergy. ACI International **14**, 204-215 (2002).
5. Gamboa, P et al. The flow-cytometric determination of basophil activation induced aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is useful for in vitro diagnosis of the NSAIDs hypersensitivity syndrome. Clin Exp Allergy **34**, 1448-57 (2004)
6. Uguccioni, M., C. R. Mackay, et al.. "High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines." J Clin Invest **100**(5): 1137-43 (1997)
7. Ducrest, S., F. Meier, et al. (2005). "Flowcytometric analysis of basophil counts in human blood and inaccuracy of hematology analyzers." Allergy **60**(11): 1446-50.

Flow CAST®

Labeled tubes

↓

50 µl Stimulation Buffer, Control or Allergen
add 100 µl Stimulation Buffer
add 50 µl whole blood

↓ *mix gently*

add 20 µl Staining Reagent

↓ *mix gently and incubate for 15 min at 37°C (waterbath)*

add 2.0 ml pre-warmed (18-28°C) Lysing Reagent

↓ *incubate 5 - 10 min at 18-28°C*

↓ *centrifuge 5 min at 500 x g and decant or aspirate supernatant*

add 300 µl Wash Buffer*

↓ *vortex gently*

Proceed to analysis by Flow Cytometry (within 8 hours)

TIME TO RESULT: ~ 1 HOUR

*** Note:** Depending on Flow cytometry instrumentation more wash buffer (e.g. 800 µl) might be necessary.

SYMBOLS/SYMBOLE/ SYMBOLES/SIMBOLI/ SIMBOLOS

Symbol	Explanation
	Use By Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
REF	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
LOT	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
	Contains sufficient for <n> tests Ausreichend für „n" Ansätze Contenu suffisant pour „n" tests Contenuto sufficiente per „n" saggi Contenido suficiente para <n> ensayos
	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura

Symbol	Explanation
BUF STIM	Stimulation Buffer Stimmulations-Puffer Tampon de stimulation tampone di' stimulazione Tampón de estimulación
CONTROL STIM	Stimulation Control Stimulationskontrolle Contrôle de stimulation Controllo di stimulazione Control de estimulación
CONTROL FMLP	Stimulation Control fMLP Stimulationskontrolle fMLP Contrôle de stimulation fMLP Controllo di stimulazione fMLP Control de estimulación fMLP
REAG STAIN	Staining Reagent Färbe-Reagenz Réactif de coloration Reagente di colorazione Reactivo de coloración
REAG LYS	Lysing Reagent Lyse Reagenz Réactif de lyse Reagente di lisi Reactivo de lísis
BUF WASH	Wash Buffer Wasch-Puffer Tampon de lavage tampone di lavaggio Tampón de lavado