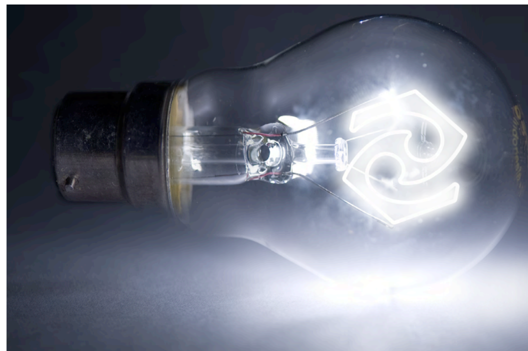
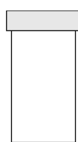
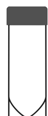


TECHNICAL BULLETIN

FSL-RFG(Maleimide) FSL Construction Kit

www.kodebiotech.com

Kit catalogue number		960819-1-R&D
FSL construct full name:		FSL-RFG(Maleimide)-SC2-L1 (cat# 033106-1-R&D)
FSL short name:		FSL-RFG(Mal)
FSL catalogue number:		033106
Components in this kit:		All reagents are sealed in a single tamper-proof outer. Keep this outer for protecting the reaction from light (step 11)
		1 × 1 mg of FSL-RFG(Mal) – (0.5 mmol) – supplied in a 2 mL polypropylene screw top vial (cat# 033106-1-R&D)
		1 × 1 mL vial of 0.1M 4MMF buffer (containing 0.1 mmol of 4-methylmorpholine formate in 30% IPA, pH 6.5-6.7 under a nitrogen atmosphere). Vials have Teflon lined lids (cat# 833913-1-R&D)
		1 × 1.5 mL empty clear screw top glass vial with PTFE liners.
Storage		Use reagents immediately once opened, dispose of unused contents. Store unopened kits at below 0^oC, preferably at minus 18^oC or below.

PRODUCT DESCRIPTION

The FSL-RFG(Maleimide) FSL Construction Kit is for use in creating Function-Spacer-Lipid (FSL) constructs for use in non-covalent cell-surface modification/engineering of cellular membranes, viral particles, liposomes, or other surfaces [1-10]. FSL-RFG(Mal) is one of several FSL constructs with Reactive Functional Groups (RFG); with this construct having maleimide as its Function group. The semi-rigid Spacer in this molecule is constructed via modified hexapeptide unit $(Gly-Gly-Ida)_2$ coupling to both amino groups of ethylenediamine and has been designed to ensure accessibility for target binding/external interactions and proper presentation of functional peptides at a cell or virion surface as well as imparting good solubility to the construct. Electrostatic repulsion forces of spacer's anionic groups probably favor uniform distribution of the incorporated constructs on the membrane surface [11]. The diacyl phospholipid derived from unsaturated fatty acids is a prerequisite for spontaneous incorporation into cell membranes. This FSL-RFG(Maleimide) FSL Construction Kit cat # 960819-1-R&D (includes a detailed procedure and contains reagents sufficient for one FSL preparation on a milligram scale from cysteine-containing peptides (Figure 1), proteins or any other thiols of biological interest. The effective synthetic approach is based on the well-known Michael nucleophilic addition to maleimides, which react fast and selectively with SH-groups in the pH range 6.5-7.5 producing stable thioether linkages completely stable at physiological conditions [12-15]. The reaction half-life between millimolar concentrations of maleimide and thiol is estimated to be of the order of few seconds [14,15]; but more complex and heavy molecules of biochemical

interest interact somewhat slower even when applied in 10-fold excess and durations of at least 2 hours are recommended [16]. The protocol described here is optimized for this kit using FSL-RFG(Mal) with generic peptides and addresses problems which may be encountered if purification of completed FSL constructs is required.

The features of the procedure include using:

- volatile buffer solution based on 4-methylmorpholine formate/30% IPA with a pH range of 6.5-6.8 to minimize both reaction non-specificity and hydrolytic degradation of FSL-RFG(Mal); the buffer also proved to be an excellent solvent for FSL-RFG(Mal), the vast majority of FSL constructs (**II**) and parent middle-sized peptides studied so far.
- a 100% molar excess of >95% HPLC pure cysteine-containing peptides or sulfhydryl-haptens over FSL-RFG(Mal) – see also Application Note #1
- straight forward removal of residual peptides during cell washing step following FSL construct incorporation or direct purification of constructs by simple re-precipitation

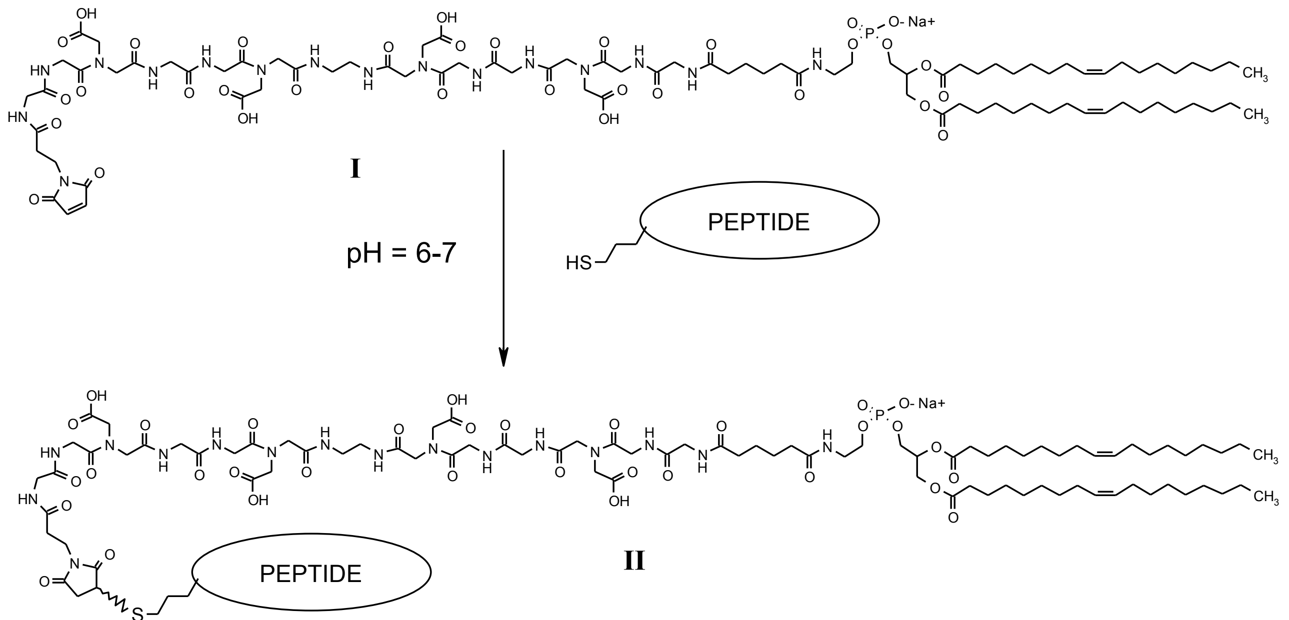
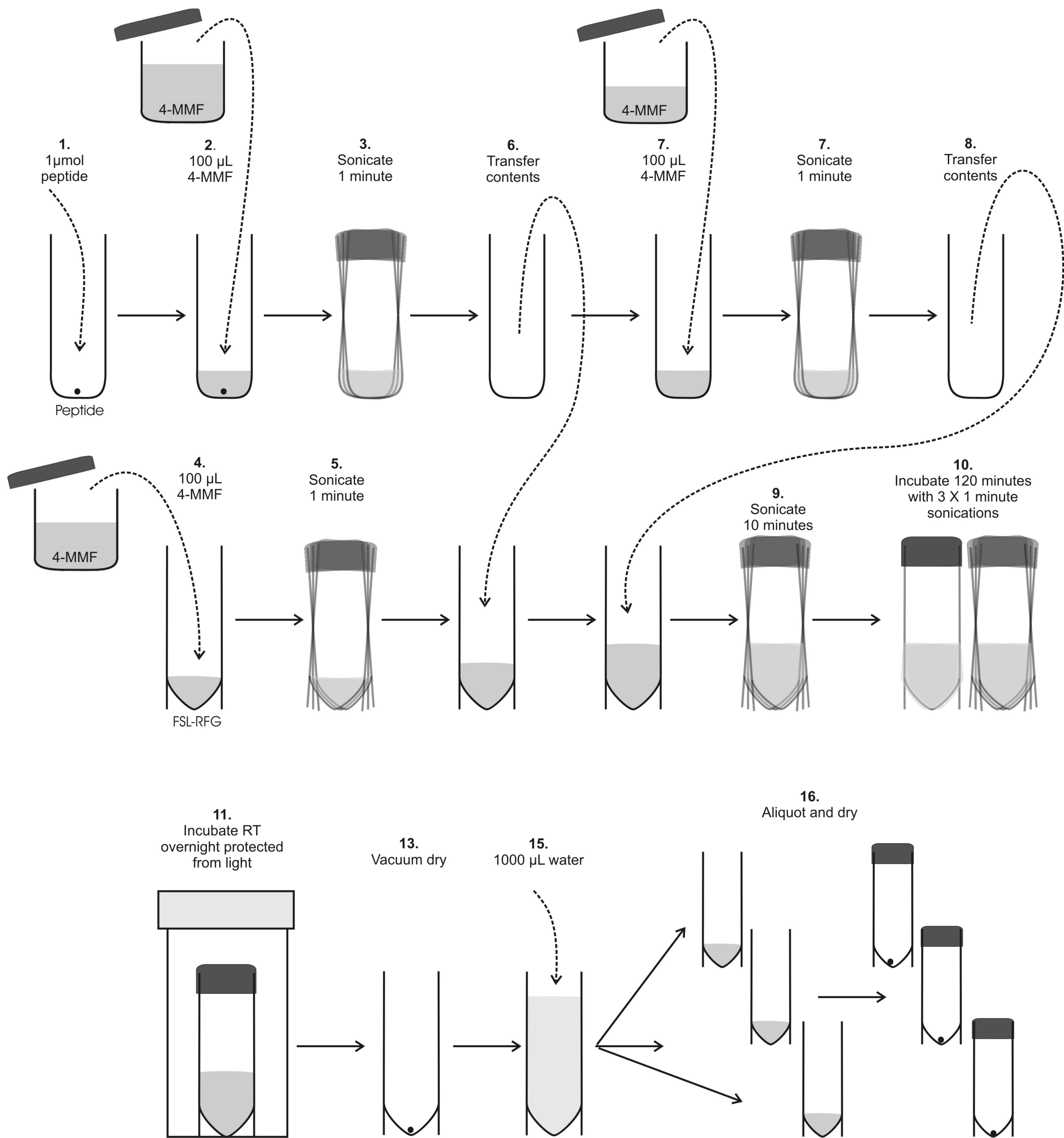


Figure 1: Formation of FSL-peptide construct **(II)** via ligation of reactive maleimide moiety of FSL-RFG(Mal) (**I**) to thiol function of cysteine incorporating peptide.

METHOD OVERVIEW (minimal procedure - Step relate directly to steps in the following method notes)



METHOD

NOTE: Read peptide consideration notes (below) before selecting peptides for ligation

1. Weigh a quantity of peptide (HPLC purity >95%) corresponding to 1 mmol (100% molar excess with respect to FSL-RFG(Mal) – Application Note #1) into the empty transparent 1.5 mL glass vial supplied.
2. Dissolve the peptide in minimal volume of 4-MMF buffer from a freshly opened vial starting from 100 mL (Application Note #2).
3. Sonicate until a clear solution is obtained and if required centrifugate briefly to remove/recover/utilize any material adhered to the tube walls. Complete dissolution of peptide is pre-requisite for successful ligation. If peptide is still not solubilized after 10 minutes in an ultrasonic bath, and the addition of further 200 mL of 0.1 M 4MMF is not helpful you may need to use one of the organic co-solvents as described in Application Note #2.
4. Reconstitute the vial of FSL-RFG(Mal) reagent (0.5 mmol) with 100 mL of 4-MMF from the freshly opened vial.
5. Sonicate or vortex for 1 minute. Use immediately.
6. Immediately transfer with a pipette the peptide solution to the reconstituted FSL-RFG(Mal).
7. Rinse the peptide tube with 100 mL of buffer, briefly sonicate
8. Transfer the rinsing's to the FSL-RFG(Mal) vial and firmly replace the cap.
9. Sonicate the reaction mixture for 10 mins (but no longer than 60 mins) in an ultrasonic bath.
10. Incubate the reaction mixture for 2 hours with 1 minute sonication approximately every 30 minutes. This step is not required if a clear solution is obtained, yet sometimes the construct partially precipitates (Application Note #3) and forms a cloudy solution or fine suspension.
11. Incubate the reactants overnight at room temperature (protected from direct light). To protect from light preferably use the outer plastic container (with lid) the kit was supplied in, or use aluminum foil.
12. Optionally quench any unreacted maleimide (See Application Note #4)
13. Dry the reactants in a vacuum centrifuge. (See Application Note #5). Alternatively you may freeze-dry the contents after transfer to a larger vial, adding 500 mL of water, immediately freeze and lyophilize.
14. The product should appear as a transparent film but if the product appears to be wet then redissolve with 500 mL of water, sonicate and dry again.
15. Reconstitute the product (containing ~0.5 mmol of FSL-peptide + ~0.5 mmol peptide) with 1 mL water.
16. Aliquot then dry into vials. The product obtained will be an amorphous white powder or clear film that is the fully functional / ready-to-use form of constructs **II**. The product should be stable if stored freeze-dried at -10°C or below for at least 10 months. Reconstituted product is relatively stable in saline. Solubility profile (see Application Note #6)

The crude FSL-peptide product is virtually free from contaminants (see also Application note #5) other than residual Cys-containing peptide, and respective SS-dimer, which will not interfere with most of the expected applications of the constructs. The final FSL-peptide product will purify itself during the cell insertion process, as only the FSL constructs are likely to insert and remain in the cell membrane. However, the user may optionally further purify the product as follows by three rounds of reprecipitation. This requires ***completely dissolving the solids*** *to remove impurities which are occluded/trapped inside the amorphous solid in a minimum of water with the help of IPA to facilitate wetting of the precipitate. This is followed by precipitation with about 10 volumes of IPA and a final EtOH wash, which efficiently removes the residual IPA with dissolved impurities (in practice, alcohol-soluble constructs are occasionally encountered; these should be precipitated with neat acetonitrile). At least one re-precipitation round should be carried out according to recommendations below to remove traces of any occluded/co-precipitated non-peptide material.*

Optional further purification

1. Sonicate the dry residue twice with IPA (100 mL) until a fine suspension is obtained and centrifuge. The FSL-peptide constructs should be in the precipitate. Do not discard the supernatants but transfer them to a 10 mL clean vial marked "Combined Washes" (Application Note #7).
2. Dissolve the precipitate in minimal volume of water (1 drop) and precipitate it with 1 mL of IPA. Centrifugate and decant the supernatant to "Combined Washes". Wash the precipitate twice with 100mL of EtOH and transfer supernatant to "Combined Washes". Repeat this wash step one further time. Transfer all supernatants to vial marked "Combined Washes.

3. Dissolve the final precipitate (expected to contain the FSL constructs) in 1 mL of water and freeze dry or vacuum centrifuge.
4. As a safeguard, we recommend to dilute Combined Washes with 2-3 volumes of water and lyophilize. The dry residue normally contains unreacted peptide material and minor by products, yet occasionally may retain considerable amounts of FSL constructs. The latter could be recovered *via* re-precipitation using alternative solvents (see Application Note #6).
5. At your convenience reconstitute the product with water and aliquot as necessary before final freeze-drying. Product may be unstable in water (at pH above 7, in particular) so lyophilize immediately after reconstitution. The product obtained will be an amorphous white powder that is fully functional /ready to use form of constructs **II**. It should be stable upon storage at -10°C or below for at least 10 months. Reconstituted product is relatively stable in saline. Solubility profile (see Application Note #6).

Chemically defined water-soluble FSL constructs produced in this way can be purified by repeated precipitation and characterized using ESI MS in negative mode and ^{1}H-NMR spectroscopy (see Application Note #10).

Reagents and Equipment Required but Not Provided (excluding optional steps)

Ultrasonic bath/sonicator; Freeze-dryer or Vacuum Centrifuge

Precautions and Disclaimer *This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.*

PEPTIDE CONSIDERATIONS FOR CONSTRUCTION INTO FSL-PEPTIDES

Once candidate peptide sequences have been identified, they should be refined to find those most likely to be compatible with, and successful as, an FSL construct. Some of these issues relate to the peptide [19,20] while others are specific to FSL chemistry. Many of the issues and characteristics of proteins/ peptides/amino acids [21-28] can be identified by using existing algorithms/rules as found in resources such as: I-Tasser [29], BLAST [30], Immune Epitope Database (IEDB, www.iedb.org) [31], hydrophilicity [32], secondary structure [23], B-cell epitope [33], flexibility [34], surface accessibility [35], N-glycosylation [37], O-glycosylation [37], etc.

Specific issues in the design and construction of peptide based FSLs include those listed below. These issues for consideration, where possible should be mitigated for, or compromises found. However in many cases they have to be simply accepted as risks, with the outcome ultimately determined by success in both synthesis and biology.

Peptide length - In theory the minimum number of amino acids required for antibody recognition is 4-6 [38]. But in practice anything less than 12 is not recommended as the additional flanking amino acid residues may also contribute to adequate epitope presentation and specificity, and increase the range of recognition by polyclonal antibodies.

Internal cysteine - peptide to spacer ligation chemistry is thiol based and a cysteine residue is used to conjugate peptide to the spacer (S) (Fig. 1). This is normally achieved by the addition of a cysteine residue to the final peptide sequence at either the amino or carboxyl ends (depending on orientation required). In principle, this limitation requires that cysteine(s) be absent from the selected peptide sequence (although this limitation could be resolved either *via* iso-steric substitution of Cys for α-aminobutyric acid [39], or potentially by using the native cysteine side chain for SL ligation).

N-terminal glutamine **-** Peptides bearing N-terminal glutamine (Gln) are known to undergo notoriously fast cyclization to form pyroglutamic acid residues [40] with the cyclization rate being in the range ~2-3% /h under physiological conditions [41]. This spontaneous transformation is likely to effect antigenicity and should be excluded where the predicted epitope begins with N-terminal Gln. Sacrificing it, or selecting peptide with an extra residue preceding Gln will remove this potential complication**.**

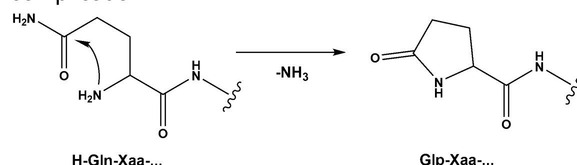


Fig. 2 Cyclization of N-terminal glutamine resulting in undesired pyroglutamic acid

Asparagine deamidation - Peptides incorporating asparagine (Asn) followed by a non-hydrophobic residue -Asn-Xaa- are prone to spontaneous deamidation via intermediate formation of 5-membered aspartimide, giving rise to a mixture of related α - and β -aspartyl peptides [42] potentially having altered antigenic profiles. The rate of degradation depends largely upon Xaa structure and is highest for Gly approaching ~2%/h under physiological conditions [43]. This type of intrinsic peptide instability is incurable by sequence manipulation and should be considered seriously. At least Asn-Gly should be excluded by all means while the potential problems in case of other Xaa should be evaluated on the basis of published data obtained for related model peptides [43] and experimental outcomes.

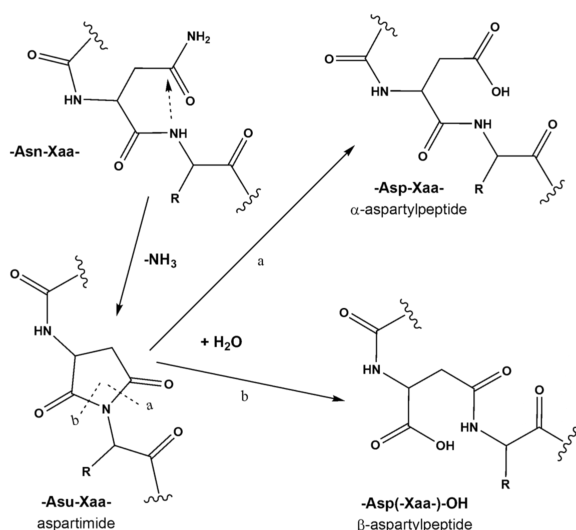


Fig. 3 Asparagine deamidation pathways resulting in undesired related α - and β -aspartyl peptide

Solubility - Perhaps the most important practical aspect of peptide selection focuses on detection and troubleshooting solubility problems associated with a particular sequence as such, and the related FSL construct. Ideally, to ensure smooth and efficient ligation, both should possess solubility in ligation buffer (0.1M 4-methylmorpholine formate in 30% isopropanol, pH~6.5) on the order of few mg/mL. Speaking from experience, we recommend avoiding sequences marked with the following insolubility signatures:

- peptides either having net charge $|Z_{pept}|<2$ themselves, or yielding FSL constructs with net charge in that region, i.e. $|Z_{pept}-5|<2$; $Z_{pept} = \Sigma(R+K+N\text{-terminus} + 0.5{*}H) - \Sigma(D+E+C\text{-terminus})$; in other words these conditions roughly specify molecules having pI and, hence, solubility minimum in physiological pH range.
- sequences harboring clusters formed by non-charged residues which include 3 or more consecutive I, F, Y, W, L, V, T

In cases when solubility improvement through sequence manipulation is impossible, co-solvents can be used to solubilize peptide prior to ligation: (i) trifluoroethanol (or hexafluoroisopropanol, or 2-methoxyethanol) - ligation buffer or pyridine (1:1, v/v), (ii) neat dimethylformamide or (iii) 6M guanidinium hydrochloride (should be tested in this order) and may prove useful for dissolving those peptides at a concentration of few mg/mL. Dimethyl sulphoxide (DMSO) may promote disulfide formation and should not be used to dissolve Cys-containing peptides. The resultant FSL construct may be more soluble than the starting peptide and although forcing conditions (e.g. increasing pH with ammonium bicarbonate, or using initially neat DMSO, glycerol, 6M urea, 30-50% aqueous alcohols followed by dilution with application-compatible medium) can allow for reconstitution of poorly soluble FSL constructs. The most important biological applications, however, ultimately require FSLs constructs dispersible in saline alone as it is this feature that allows modification of cells without affecting their vitality and functionality. In this connection, preliminary evaluation of potential insolubility issues is strongly recommended to avoid wasting efforts on preparation of insoluble and useless material.

Potential glycosylation sites - Both N- and O- potential glycosylation needs to be considered during the peptide selection process. The standard N-glycosylation sequence is N-X-S/T where X-could be any amino acid other than proline [44]. There is no single O-glycosylation motif but it usually occurs with high content of serine, threonine and proline residues [36] and in combinations like TAPP, TVXP, S/TPXP, TSAP, PSP, and PST, where X is any amino acid [44]. As glycosylation dramatically changes the nature of an epitope the following strategies should be considered: (i) optimizing the peptide to avoid residues suspected in glycosylation or (ii) direct chemical synthesis of appropriately glycosylated peptides [45] or as a last resort (iii) using a naked peptide in the hope that it may still retain the expected binding specificity.

Needless to say, these considerations also apply to any post-translational modification [46,47] in the proximity of the epitope sequence.

Microbial relatedness - algorithms for comparing primary biological sequences to microbial sequences (e.g. BLAST [30], Immune Epitope Database [31]) help predict and evaluate the degree of potential cross-reactivity associated with the proposed peptide. Avoidance of microbially related epitopes reduces the risk of undesired non-specific cross-reactivity from

naturally occurring antibodies directed against microbes.

PREPARATION OF CELLS/VIRIONS MODIFIED WITH FSL-PEPTIDE CONSTRUCTS [1]

Typically biological activity is obtained with peptide-based FSL constructs in the range of 1-100 μg of FSL construct per mL of cell transformation solution. The working range dilutions will depend on the application, the detection method sensitivity, the diluent, the type of FSL construct, and the degree of modification required.

Preparation of FSL Constructs

i. Prepare the FSL construct stock solution by reconstituting the dry FSL product by the addition of appropriate diluent (preferably not containing lipids or highly hydrophobic material). Briefly sonicate (30 seconds). FSL constructs can be diluted in water but will have reduced stability and must be used within hours.
ii. Prepare working FSL construct solutions for insertion, just prior to use. Briefly sonicate (30 seconds) the stock solution to homogenize any micelles. Dilute the FSL construct in buffer (preferably not containing lipids or highly hydrophobic material) to the concentration required or over a range if desired. FSL constructs will usually insert into cells in lipid containing media but will typically require as much as a 50 × higher FSL concentrations than if in PBS or other lipid free media.

Insertion of FSL Construct(s) into Membranes

iii. Wash the cells for FSL modification free of unbound lipids by centrifugation and using PBS or lipid free cell media as wash solution. The diluent used to suspend the cells can be cell culture media, PBS, cell storage solutions, etc, but preferably without lipids (e.g. fetal calf serum) or detergents (e.g. Tween).
iv. Suspend in 100μL of diluent. Different volumes (than 100μL) or ratios (than 1:1 cells/FSL solution) may be used provided the same ratio, concentration, and volume are used to obtain reproducible results.
v. Add 100μL of an appropriate dilution of FSL solution (containing 1 or more FSL constructs) to the cells and incubate for 1 hour at 37°C. A similar result can be obtained by 6 hours incubation at 25ºC or overnight (18 hours) at 4ºC – mixing is recommended every few hours if heavy cell suspensions are used.
vi. Wash (optional) twice to remove any free FSL constructs and prepare an appropriate cell suspension.

APPLICATION NOTES

Note #1. This procedure can be done with only a 20% molar excess of >95% HPLC purity peptides. However, despite claims of >95% purity, the quality and purity of peptides supplied by various vendors is variable [48] and together with risks of cysteine residue oxidation and small volume weighing errors, this method advises use of 100% molar excess of peptide. If 100% excess is used then the user can be confident the reaction will go through to completion.

If the user elects to use a 20% molar excess then exact calculation of the stoichiometric quantity of a synthetic cysteine-containing peptide is required. The most professional approach to calculate the adequate quantity implies spectrophotometric determination of actual reactive thiol content C_{SH} in mmol HS/mg in peptide material to be used. Standard procedures employing DTNB [17] (Ellman reagent) detailed in Sigma Technical Bulletins D8130 and MBK1 are recommended. In case the determined HS-content is too low, the reduction of disulfide may be in order according to one of the available protocols [18]. Despite popular belief this problem could not be fixed by performing ligation in the presence of tributylphosphine or TCEP which were recently shown to react with maleimides [49,50]. Weight of peptide material required for 1 ligation (0.6 mmol, 20% molar excess) is calculated as W, mg = $0.6/C_{SH}$

In case spectrophotometric quantitation of thiol content is not performed, the required peptide quantity could be calculated assuming peptide content in an average HPLC-purified synthetic peptide material to be 75% [48] as a best approximation. Within this approach the weight of peptide material required for 1 ligation (0.6 mmol, 20% molar excess) is calculated as W, mg=0.6*1.2*MW/0.75= 0.96*MW, where MW stands for nominal molecular weight of the peptide (without counter-ions) expressed in mg/mmol.

Note #2. Reaction buffer 0.1M 4MMF vials are supplied degassed and in a nitrogen atmosphere. Use a freshly opened vial for each ligation reaction.

Checking the extent of peptide solubility in the ligation buffer before hand is strongly recommended (see *Solubility* notes above). The vast majority of middle-sized peptides will dissolve at concentration 5-3 mg/mL and for these this protocol will work consistently.

Peptides that are not completely soluble at 1 mg/mL are unlikely to respond to further increasing buffer to peptide ratio. *In many cases satisfactory results will be obtained with peptides insoluble in the ligation buffer at 1mg/mL and hence need to be introduced into reaction dissolved in organic or chaotropic medium. The success of such experiments is not certain and these may be undertaken at user's own risk.*

Note #3. Poor solubility can be expected for constructs **II** with overall charge in the range -2 ÷ +2, i.e. those derived from basic peptides bearing net electric charge +4 ÷ +6

Note #4. Optionally quench any residual maleimide on FSL-RFG(Mal) with a suitable non-interfering thiol (cysteine, DTT, ME, etc) before freeze drying . For example mix thoroughly reaction mixture with 60 mL of 0.1M mercaptoethanol and leave it for at least 1 hour at room temperature to ensure complete quenching of any unreacted maleimide component **I**.

Note #5. A simplified precipitation step may be introduced here to be sure that user has completely removed the 4-MMF salt at the end of vacuum drying. This salt is the least volatile component that may persist at the transparent film stage in the amounts harmful to you cells. At least single precipitation (IPA or MeCN) is highly desirable to ensure 4-MMF is no longer an issue. To do this sonicate thoroughly the crude product obtained as a transparent film with 300 mL of IPA, decant the fine suspension thus obtained and repeat the IPA wash. Redissolve the wet product in a minimum amount of water (~50 mL) and precipitate with 10-15 volumes of acetonitrile, centrifugate, wash with 200 mL of IPA and dry in vacuum. Drying compounds II using Fisher apparatus at elevated temperatures, or by washing with copious amounts of absolute ether, albeit highly efficient, should be avoided since both of them yield over-dried material that is extremely difficult to reconstitute in water.

Note #6. These recommendations are based on generalized solubility profile typical of constructs **II** derived from water soluble peptides:

- Solubility in unbuffered water is about 1-5 mg/mL and could be increased 2-3-fold by mild basification (ammonium bicarbonate solution, or pyridine); not unexpectedly, acidifying produce an opposite effect and precipitates **II** from an aqueous solution. The indicated pH-dependence reflects domination of acidic residues in molecules of this group. However, the reversal of the trend should be expected and was actually observed for constructs derived from peptides bearing overall charge of +6 and more
- Product may be unstable in water for extended periods of time, and should only be used as a working solution in saline solutions or solvents.
- in 10-50% unbuffered IPA - up to 10-20 mg/mL (optimal % of IPA depends on sequence; this alcohol appeared to be most compatible with compounds **II** and should be preferred to MeOH & EtOH);
- in DMSO, DMF, NMP - 10-20 mg/mL and above;
- in acetone, MeCN, dioxan, THF, IPA, EtOH - very poor, but increases with increase of water content;
- insoluble in Et_2O, EtOAc, DCM and other solvents immiscible with water.

Note #7. As a safeguard, collect all washes generated in the course of isolation and keep them combined in an appropriate PP tube. This precaution is necessary in a view of unpredictable solubility behavior of constructs **II** derived from diverse peptides, which makes impossible to formulate a universal isolation protocol devoid of the risk of discarding the desired product. In any case, a second crop of **II** could be obtained via acetonitrile precipitation from the vacuum-evaporated combined washes.

Note #8. TLC. Presence of polyanionic spacer in **I** and its derivatives **II** results in anomalous chromatographic behavior of these compounds. They are not eluted from RP HPLC column under standard binary gradients of MeOH or MeCN, even on prolonged elution. In principle, at least for some peptides, TLC in one of the following solvent systems may be useful.

- $CHCl_3$/MeOH/H_2O 1:3:1 (v/v+0.5% pyridine),
- $CHCl_3$/MeOH/H_2O 2:6:1 (v/v+0.5% pyridine)
- MeCN:MeOH:H_2O, 3:3:2, v/v

and staining with 0.3% ninhydrine in 3% AcOH-n-BuOH, or 7% H_3PO_4 in IPA

But, in reality many peptides will require laborious experimental selection of optimal sequence-specific solvent system to obtain a clear-cut TLC pattern. The efficiency of the present ligation protocol resides, therefore, on carefully optimized reaction conditions rather than real-time analytical control of the reaction.

Note #9. Spectroscopic characterization of constructs **II**. Unique but unfavorable from practical point of view solubility profile of **II** makes them not compatible with RP HPLC, while on the other hand, natural diversity of parent peptides makes potential usage of otherwise straightforward TLC analysis highly problematic. Using

modern spectroscopic techniques like NMR or MS generates important structural evidence for **II** but within certain reservations that need commenting. Most straightforward and reassuring evidence for **II** was offered so far by mass-spectrometry with **ESI** ionization **specifically** and in **negative** mode ONLY, with **direct injection** of sample solution being an extra strict requirement that guarantees recording most adequate and informative spectrum. The desired molecules **II** are represented in the latter by the expected set of clusters formed by multiple charged anions derived from molecular ion. These consistently predominate over minor peaks of residual SS-dimer of the starting peptide and secondary fragmentation peaks. Curiously, the latter formally correspond to masses of starting reagents: maleimide **I** and the reduced peptide thiol, the artifacts of retro-Michael fragmentation, which could be suppressed by adjusting ionization voltage of the ion source. Occasionally performed parallel analyses of the same samples using ESI and more destructive MALDI technique (positive mode) suggested that the latter is not compatible with FSL constructs since it generally fails to detect even traces of expected molecular ions and produces non-informative and misleading patterns.

^{1}H-NMR spectra recorded for solutions of **II** in 20-60% CD_3OD/D_2O could provide further qualitative support for the expected structure, with the low-field region (starting from ~4 ppm downfield) being most informative. Quantitation of NMR spectra in a usual way is not possible because formation of micelles/aggregates interferes with relaxation phenomenon and distorts integral intensities.

REFERENCES

1. Blake DA, Bovin NV, Bess D, Henry SM: FSL Constructs: A simple method for modifying cell/virion surfaces with a range of biological markers without affecting their viability. *J Visualized Experiments* 2011; (in press) doi: 10.3791/3289
2. Hult AK, Frame T, Chesla S, Henry S, Olsson ML: Flow cytometry evaluation of red blood cells mimicking naturally-occurring ABO subgroups following modification with variable amounts of FSL-A and B constructs. *Transfusion* 2011; (in press) doi: 10.1111/j.1537-2995.2011.03268.x
3. Frame T, Carroll T, Korchagina E, Bovin N, Henry S: Synthetic glycolipid modification of red blood cell membranes. *Transfusion* 2007; 47:876-882
4. Oliver C, Blake D, Henry S: In vivo neutralization of anti-A and successful transfusion of A antigen incompatible red cells in an animal model. *Transfusion* 2011; (accepted) doi: 10.1111/j.1537-2995.2011.03184.x
5. Oliver C, Blake D, Henry S: Modeling transfusion reactions and predicting in vivo cell survival with kodecytes. *Transfusion* 2011; (accepted) doi: 10.1111/j.1537-2995.2010.03034.x
6. Henry S: Modification of red blood cells for laboratory quality control use. *Curr Opin Hematol* 2009; **16**:467-472
7. Heathcote D, Carroll T, Wang JJ, Flower R, Rodionov I, Tuzikov A, Bovin N, Henry S: Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto erythrocytes. *Transfusion* 2010; **50**:635-641
8. Georgakopoulos T, Komarraju S, Henry S, Bertolini J: An improved Fc function assay utilizing CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs. *Vox Sang* 2011; (accepted) doi: 10.1111/j.1423-0410.2011.01512.x
9. Harrison, AL, Olsson ML, Brad Jones R, Ramkumar S, Sakac D, Binnington B, Henry S, Lingwood CA, Branch DR: A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms. *Glycobiology* 2010; **27**:515-524
10. Hadac EM, Federspiel MJ, Chernyy E, Tuzikov A, Korchagina E, Bovin NV, Russell S, Henry SM. Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple in vitro and in vivo bioimaging of enveloped virions *J Virol Meth* 2011 (accepted) doi:10.1016/j.jviromet.2011.06.005
11. Biobased Surfactants and Detergents - Synthesis, Properties, and Applications. Hayes, D.G., Kitamoto, D., Solaiman, D.K.Y., Ashby, R.D. (Eds). AOCS Press 2009, p.361.
12. Smyth, D.G., Nagamatsu, A., and Fruton, J.S. Reactions of N-ethylmaleimide . J. Am. Chem. Soc. 1960, 82, 4600.
13. Smyth, D.G., Blumenfeld, O.O., and Konigsberg, W. Reaction of N-ethylmaleimide with peptides and amino acids. Biochem. J. 1964, 91, 589.
14. Gorin, G., Martin, P.A., and Doughty, G. Kinetics of the reaction of N-ethylmaleimide with cysteine and some congeners. Arch. Biochem. Biophys. 1966, 115 , 593
15. Bednar RA. Reactivity and pH dependence of thiol conjugation to N-ethylmaleimide: detection of a conformational change in chalcone isomerase. Biochemistry. 1990, 29(15), 3684-90.
16. Bioconjugate Techniques. Hermanson, G.T. (Ed). Academic Press-Elsevier 2008, p.161
17. Ellman GL. Tissue sulfhydryl group. Arch. Biochem. Biophys. 1959; 82: 70–77.
18. Reversal of Inadvertent Oxidation of Cys-containing Peptides. BACHEM Technical note 2007.
19. Topp EM, Zhang L, Zhao H, Payne RW, Evans GJ, Manning MC: Chemical Instability in Peptide and Protein Pharmaceuticals; in Jameel F, Hershenson S (eds): Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals. New Jersey, John Wiley & Sons, Inc., 2010
20. Li S, Schoniech C, Borchardt RT: Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization; in Papoutsakis ET (eds), Biotechnology and Bioengineering. New Jersey, John Wiley & Sons, Inc, 1995
21. van Regenmortel MHV, Muller S: Synthetic peptides as antigens; in Pillai S, Van der Vliet PC (eds): Laboratory Techniques in Biochemistry and Molecular Biology, v.28. Amsterdam, Elsevier, 1999
22. van Regenmortel, MHV: What Is a B-Cell Epitope?; in Schutkowski M, Reineke U, (eds): Methods in molecular biology, *Epitope Mapping Protocols*. New York, Humana Press, 2009
23. Fasman GD, (ed): Prediction of Protein Structure and the Principles of Protein Conformation. New York, Plenum Press, 1989
24. Zegers ND, Boersma WJA, Claassen E (eds): Immunological Recognition of Peptides in Medicine and Biology. New York, CRC press, 1995
25. Celada F, Schumaker VN, Sercarz EE (eds): Protein conformation as an immunological signal, EMBO. New York, Plenum press, 1983
26. Conte LL, Chothia C, Janin J: The atomic structure of protein-protein recognition sites. *Journal of Molecular Biology* 1999; **285**:2177-2198
27. van Regenmortel MHV: Molecular design versus empirical discovery in peptide-based vaccines. Coming to terms with fuzzy recognition sites and ill-defined structure-function relationships in immunology. *Vaccine* 1999; **18**:216-221
28. Arnon R, van Regenmortel MHV: Structural basis of antigenic specificity and design of new vaccines. *The FASEB Journal* 1992; **6**:3265-3274

29 Ambrish Roy, Alper Kucukural, Yang Zhang. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 2010; **5**:725-738

30 Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden T: BLAST+: architecture and applications. BMC Bioinformatics 2009; **10**:421-9

31 Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, Damle R, Sette A, Peters B: The Immune Epitope Database 2.0. *Nucleic Acids Res* 2010; **38**:D854-D862

32 Parker JM, Guo D, Hodges RS: New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry* 1986; **25**:5425-5432

33 Larsen JE, Lund O, Nielsen M: Improved method for predicting linear B-cell epitopes. *Immunome Res* 2006; **2:2**:1-7

34 Karplus PA, Schulz GE: Prediction of chain flexibility in proteins: A tool for the selection of peptide antigens. *Naturwissenschaften* 1985; **72**:212-213

35 Petersen B, Petersen TN, Andersen P, Nielsen M, Lundegaard C: A generic method for assignment of reliability scores applied to solvent accessibility predictions. *BMC Struct Biol* **2009**, **9**:1-10

36 Gupta R, Brunak S: Prediction of glycosylation across the human proteome and the correlation to protein function. *Pac Symp Biocomput* 2002; **7**:310-322

37 Julenius K, Molgaard A, Gupta R, Brunak S: Prediction, conservation analysis, and structural characterization of mammalian mucin-type O- glycosylation sites. *Glycobiology* (2005); **15**:153-164

38 Tang XL, Tregear GW, White DO, Jackson DC: Minimum requirements for immunogenic and antigenic activities of homologs of a synthetic peptide of influenza virus hemagglutinin. *J Virol* 1988; **62**:4745-4751

39 Wlodawer A, Miller M, Jaskolski M, Sathyanarayana BK, Baldwin E, Weber IT, Selk LM, Clawson L, Schneider J, Kent SB: Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. *Science* 1989; **245**:616-21

40 Abraham GN, Podell DN: Pyroglutamic acid. Nonmetabolic formation, function in proteins and peptides, and characteristics of the enzymes effecting its removal. *Mol. Cell. Biochem* 1981; **38**(pt 1):181-90

41 Dick LW, Kim C, Qiu D, Cheng KC: Determination of the origin of the N-terminal pyro-glutamate variation in monoclonal antibodies using model peptides. *Biotechnology and Bioengineering* 2007; **97**:544–53

42 Geiger T, Clarke S: Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide linked reactions that contribute to protein degradation. *J Biol Chem* 1987; **262**:785–94

43 Robinson NE, Robinson AB (eds): Molecular Clocks: Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins. Cave Junction, OR, Althouse Press 2004

44 Christlet THT, Veluraja K: Database analysis of O-glycosylation sites in proteins. *Biophys J* 2001; **80**:952-960

45 Liakatos A, Kunz H. Synthetic glycopeptides for the development of cancer vaccines. *Curr Opin Mol Ther* 2007; **9**: 35-44

46 Reinders J, Sickmann A: Modificomics: Posttranslational modifications beyond protein phosphorylation and glycosylation. *Biomol Eng* 2007; **24**:169-77

47 Meri S, Baumann M: Proteomics: posttranslational modifications, immune responses and current analytical tools. *Biomol Eng* 2001; **18**:213-20

48 Cook, R. <cook@MIT.EDU>. Subject: Pep Synth- Correct method of determining peptide yield? <http://abrf.org/index.cfm/list.home>, 30 Aug 2006.

49 Shafer, D.E., Inman, J.K. and Lees, A. Reaction of Tris(2-carboxyethyl)phosphine (TCEP) with Maleimide and a-Haloacyl Groups: Anomalous Elution of TCEP by Gel Filtration. Analytical Biochemistry 2000, 282, 161–4.

50 Tyagarajan K, Pretzer E, Wiktorowicz JE. Thiol-reactive dyes for fluorescence labeling of proteomic samples. Electrophoresis. 2003 24(14), 2348-58.

DOCUMENT CONTROL

Authors: Steve Henry *(Biotechnology Research Institute, AUT University, Auckland, New Zealand)* & Igor Rodionov *(Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Pushchino, Russia)*

Correspondence: shenry@kodebiotech.com

Website: www.kodebiotech.com

Version: 20110928

Release date: 28th September 2011