STANDARD OPERATING PROCEDURE

Title: Protein Separation and Identification using Automated Western

SOP#: M-134

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1. PURPOSE

This procedure is to be used for protein separation and identification under automated conditions using Simple Western™ immunoassay technology on the Jess ™ instrument by ProteinSimple.

2. SCOPE

This document describes the Simple Western™ immunoassay procedure using the Jess™ instrument. The Jess™ instrument separates proteins based on molecular weight. Target proteins are identified using immuno- and chemiluminescence detection. Samples may be re-probed using the RePlex option.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with lab safety procedures and Compass software for Simple Western™ Assays. All procedural steps are to be followed as written and any deviations, problems and observations during an experiment must be documented. The interpretation of the results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

- Jess™ instrument; ProteinSimple (Bio-Techne).
- Centrifuge with plate adaptor (Eppendorf, Model 5810R, 15amp with swing bucket rotor or equivalent)
- Standard Heat Block Heater (VWR, Catalog #12621-104, Model 949310 or equivalent)

- Vortex

5. MATERIALS

- Jess Separation Module with EZ Standard Pack. The separation module contains capillary cartridge, pre-filled microplates, wash buffer, 10X sample buffer, and EZ Standard Pack (PS- ST01EZ-8, PS-ST03EZ-8, or PS-ST05EZ-8). Proper selection of separation module depends on the size of the target protein.
  Note: Most protein molecular weights range from 12-230 kDa.
  Available separation modules:
  - 12-230 kDa – Protein Simple, Cat # SM-W001
  - 66-440 kDa – Protein Simple Cat. # SM-W005
  - 2-40 kDa – Protein Simple Cat. # SM-W009
- Water (molecular biology grade)
- Pipettes and tips
- Micro-centrifuge tubes
- Ice and ice bucket

6. REAGENTS

For rabbit or mouse monoclonal antibody evaluation

- Protein samples (recombinant protein, over-expressed lysate, cell or tissue lysate) corresponding to the antibodies to be tested
- Primary antibodies to be tested
- Bio-Techne Anti-Rabbit or Anti-Mouse Detection Modules (respectively DM-001 and DM-002) containing Luminol-S (Cat. # 043-311), Peroxide (Cat. # 043-379), Streptavidin-HRP (Cat. # 042-414), Antibody Diluent #2 (Cat. # 042-203)
- Bio-Techne EZ Standard Pack (included in separation module SM), containing ready to use biotinylated ladder (12-230 kDa, Cat. # PS-ST01EZ-8, 66-440 kDa, Cat. # PS-ST03EZ-8, 2-40 kDa, Cat # PS-ST05EX-8), Fluorescence 5X Master Mix, and DTT.
- (Optional) Bio-Techne RePlex Module (Cat. # RP-001), containing RePlex Reagent 1 (Cat. # RP-001-1) and RePlex Reagent 2 (RP-001-2)

7. GENERAL GUIDANCE ON INITIAL TESTING AND ASSAY DEVELOPMENT

Test the antibody against purified recombinant protein, over-expressed lysate, and whole cell or tissue lysates using the following conditions:

- The antibody is initially tested at an antibody dilution of 1:25 starting from a stock antibody concentration of 1 mg/mL (equivalent to 40 ug/mL). Dilute antibody in Antibody Diluent #2. The analyte concentration varies:
  - Recombinant protein: 0.04 mg/mL
  - Overexpressed lysate: 0.1 mg/mL
  - Cell or tissue lysate: 0.4 mg/mL
- The possible outcomes of the initial testing are the following:
  - *Negative*: no signal detected at the expected MW (+/- 20%). In this case there is no need for further analysis, and the antibody is considered not suitable for this application.
  - *Positive*: peak is at the expected MW (+/- 20%), with a height less than 100,000 and baseline less than 5000 or less. In this case there is no need for further analysis, and the antibody is considered suitable for this application.
  - *Positive with high background*: peak is at the expected MW (+/- 20%), with a height greater than 100,000 or baseline greater than 5000. In this case, there is need for further analysis, and the antibody is considered potentially suitable for this application, however assay optimization is needed.

Assay optimization: When assay optimization is required, proceed to titrate the antibody and/or the analyte concentration. Titrate only one variable at a time.

- *Antibody titration range*: 1:25 to 1:1600 (assuming initial concentration of the antibody is 1 mg/mL, corresponding to a range between 40 ug/mL to 625 ng/mL)
- *Recombinant protein range*: in this case, range will vary, but the maximum concentration of the recombinant protein should never exceed 0.04 mg/mL
- *Over-expressed lysates range*: 0.005 to 0.1 mg/mL
- *Cell or tissue lysates range*: 0.05 to 1.6 mg/ml

Note: RePlex is an optional feature, in which the primary and secondary antibodies are stripped from the capillary, but the proteins are still cross-

linked to the internal wall of the capillary. After stripping, the sample can be probed again with a new set of primary and secondary antibodies. This option can be used to test different antibodies or to probe cell lysates with antibodies against house-keeping proteins or against tag, in case of tagged recombinant proteins (e.g. Myc tag). The use of RePLex reagents is described in section 8.5.

8. PROCEDURE

8.1. Preparation of Standard Pack Reagents

8.1.1 Follow Separation Module insert indications to mix Fluorescent 5X Master Mix, ladder and DTT.

8.2. Preparation of Samples

8.2.1. Follow directions in chosen Separation Module insert.

- Dilute protein samples in 0.1X Sample Buffer (Dilute 10X Sample Buffer 1:100 in H2O) for an intermediate stock (IS) concentration of 0.5 ug/uL.

- Combine 1 part 5X Fluorescent Master Mix with 4 parts IS protein preparation in a microcentrifuge tube (final concentration 0.2 ug/uL). Mix with gentle pipetting. *Example*: Add 2 uL 5X Fluorescent Master Mix to 8 uL IS protein solution

- Denature samples and biotinylated ladder at 95°C, 5 min. using the heat block heater. Then vortex, do a quick spin at Room Temperature and store on ice until ready to proceed with the microplate set-up (Step 8.6.2.1).

8.3. Preparation of Antibodies

8.3.1. Dilute the primary antibody in Antibody Diluent #2 to final concentration of 0.2 ug/mL, for a minimum final volume of 500 uL.

8.3.2. Secondary antibody (anti-mouse or anti-rabbit) is supplied with the kit and is ready to use without dilution.

8.4. Preparation of Luminol-S and Peroxide

8.4.1. Combine 200 uL of Luminol-S and 200 uL of Peroxide.

8.4.2. If running RePlex assay, a larger volume is needed, therefore combine 450 uL of Luminol-S and 450 uL of Peroxide. Dispense Luminol-S and Peroxide freshly prepared mix following RePlex Module (RP-001) insert.

- ***Note***: Luminol-S/ Peroxide mixture should be prepared immediately before use.

8.5. Preparation of RePlex Reagents (Only if running RePlex assay)

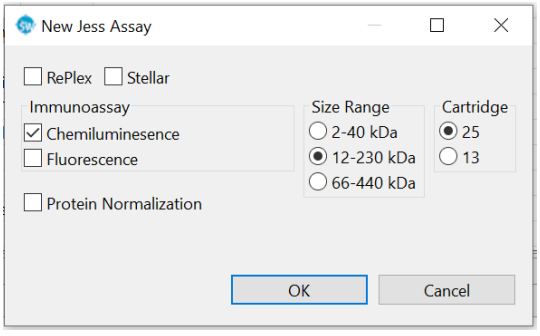
8.5.1. Combine 1.4 mL of RePlex Reagent 1 and 0.35 mL of RePlex Reagent 2 in a microcentrifuge tube. Dispense following RePlex Module (RP-001) insert.

- Note: RePlex Reagents should be prepared immediately before use.

8.6. Microplate Set -Up

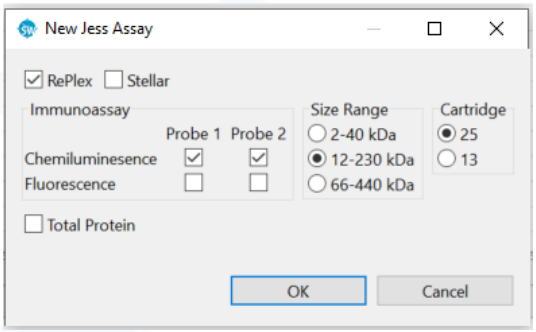
8.6.1 On the acquisition software (Compass™), from the Assay tab select File→ New Assay →Jess and indicate the immunoassay type (chemiluminescence), size range (typically 12-230 KDa), and cartridge size (typically 25).

Example:

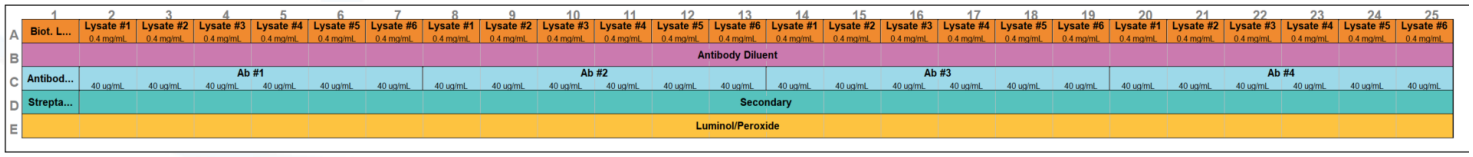


If using the RePlex option, select the RePlex in the New Jess assay tab and define the immunoassay type (chemiluminescence) for probe #1 and probe #2, size range (typically 12-230 KDa), and cartridge size (typically 25).

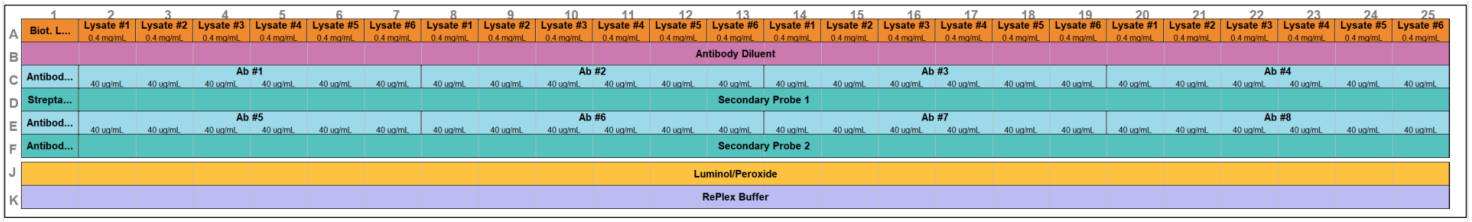
Example:



8.6.2 Use the Assay tab to define the plate layout (sample position on the plate and concentration). See template below:



When using the RePlex option, it is required to also fill probe #2 information, as in the example below:



8.6.2.1 Dispense reagents into microplate as defined above.

<u>Note</u>: This part of the procedure deviates from the insert instructions for the sample volume, loading 5 uL of sample, rather than 3 uL.

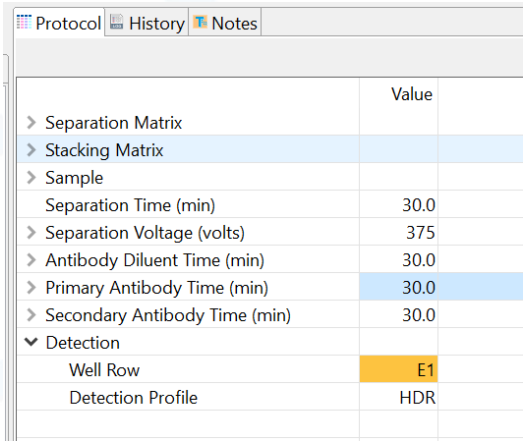
8.6.2.2 Centrifuge microplate for 5 min, @ 2500 rpm (~1000 x *g*) at room temperature. Ensure liquid is fully down in all wells.

8.7. Starting Jess Assay

8.7.1. In the Assay tab (protocol tab) set up the assay:

- Set Antibody Diluent Time to 30 minutes
- Set Separation Time to 30 minute

Example:



8.7.2. Then Open Jess' door and start Jess separation module protocol.

9. DATA ANALYSIS

Report the antibody test results as follows:

- *Negative* (not suitable for SW analysis): no signal detected at the expected MW (+/- 20%), or excessive non-specific binding detected.
- *Positive* (suitable for SW analysis): peak is at the expected MW (+/- 20%), with a height less than 100,000 and baseline of 5000 or less
- *Presumed positive* (presumably positive, but presence of extra bands): peak is at the expected MW (+/- 20%), with a height less than 100,000 and baseline of 5000 or less, although there are other bands, possibly due to non-specific binding or protein degradation.

10. REFERENCED DOCUMENT

10.1. Operation Manual, Jess™ Instrument
10.2. Operation Manual, Compass™ Software
10.3. Separation Module Protocol (SM-W001-SM-W0012)
10.4. RePlex Module Protocol (RP-001)