Instruction Sheet

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Preparation of XF Assay Media

This basic procedure details the preparation of assay media for use with XFe96/XF96, XFe24/XF24, and XFp Analyzers and the following Agilent Seahorse XF assay kits:

- Cell Mito Stress Test
- Cell Energy Phenotype Test
- Mito Fuel Flex Test
- Real-Time ATP Rate Assay
- Glycolytic Rate Assay
- Glycolysis Stress Test

The use of non- or low-buffered assay medium is recommended for XF assays to ensure accurate, consistent functional measurements of metabolic activity. The assay medium compositions are slightly different depending on the assay kit used. All compositions can be prepared using the recommended Seahorse XF Media and adding different XF supplements/buffer as determined by the specific assay design.

Please see the Agilent Seahorse XF Media and Buffer Selection Guide to determine the proper XF Seahorse media and supplements required for the XF assay to be performed: http://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf.

Agilent Seahorse XF Assay Media Preparation for: Cell Mito Stress Test, Cell Energy Phenotype Test, Mito Fuel Flex Test

	Reagent / Agilent Part Number	Final Concentration	XFe96 XF96 XFe24 (100 mL)	XF24[c] (100 mL)	XFp (10 mL)
Recommended Media[a]	Seahorse XF DMEM Medium, pH 7.4 / 103575-100 Seahorse XF RPMI Medium, pH 7.4 / 103576-100	-	97 mL	N.C.	9.7 mL
Alternative Media[b]	XF Base Medium (w/out Phenol Red) / 103335-100 XF RPMI (w/out Phenol Red) / 103336-100 XF Base Medium / 103334-100	-	97 mL	97 mL	9.7 mL
Required Supplements	Seahorse XF Glucose (1.0 M solution) / 103577-100	10 mM	1.0 mL	1.0 mL	100 µL
	Seahorse XF Pyruvate (100 mM solution) / 103578-100	1 mM	1.0 mL	1.0 mL	100 µL
	Seahorse XF L-Glutamine (200 mM solution) / 103579-100	2 mM	1.0 mL	1.0 mL	100 µL

[a]XF DMEM and RPMI Medium, pH 7.4 have a pre-adjusted pH value and do not require adjustment of pH upon addition of XF supplements. See Method 1 below for preparation

[b]XF Base Medium (without Phenol Red), XF RPMI (without Phenol Red) or XF Base Medium may be substituted for XF DMEM or RPMI Media pH 7.4. If substituted, adjustment of final media pH value to 7.4 is required. See Method 2 below for preparation.

[c]Seahorse XF DMEM Medium pH 7.4 and RPMI Medium, pH 7.4 are not compatible with XF24 Analyzers.

Agilent Seahorse XF Assay Media Preparation for: Real-Time ATP Rate Assay and Glycolytic Rate Assay

	Reagent / Agilent Part Number		Final Concentration	XFe96 XF96 XFe24[c] (100 mL)	XFp (10 mL)
Recommended Media[a]	Seahorse XF DMEM Medium, pH 7.4 / 103575-100 Seahorse XF RPMI Medium, pH 7.4 / 103576-100		-	97 mL	9.70 mL
Alternative Media[b]	XF Base Medium (w/out Phenol Red) / 103335-100 XF RPMI (w/out Phenol Red) / 103336-100		-	97 mL	9.64 mL
Alternative Media Supplement[b]	Seahorse XF 1 M HEPES / 103337-100	If using XF Base (w/o Phenol Red)	5 mM	500 µL	50 µL
		If using XF RPMI (w/o Phenol Red)	1 mM	100 µL	10 µL
Required Supplements (For Recommended and Alternative Media)	Seahorse XF Glucose (1.0 M solution) / 103577-100		10 mM	1.0 mL	100 µL
	Seahorse XF Pyruvate (100 mM solution) / 103578-100		1 mM	1.0 mL	100 µL
	Seahorse XF L-Glutamine (200 mM solution) / 103579-100		2 mM	1.0 mL	100 µL

[a]XF DMEM and RPMI Medium, pH 7.4 contain HEPES buffer at 5 and 1 mM, respectively. Both have a pre-adjusted pH value and do NOT require addition of HEPES nor adjustment of pH upon addition of XF supplements. See Method 1 below for preparation.
[b]XF Base Medium (without Phenol Red), XF RPMI (without Phenol Red) or XF Base Medium may be substituted for XF DMEM or RPMI Media pH 7.4. If substituted, addition of XF HEPES buffer to 5 mM or 1 mM, (respectively for XF Base and XF RPMI, w/o PR), and adjustment of final media pH value to 7.4 is required. See Method 2 below for preparation.
[c]The Real-Time ATP Rate Assay and Glycolytic Rate Assay are not compatible with XF24 Analyzers.

Agilent Seahorse XF Assay Media Preparation for: Glycolysis Stress Test

	Reagent / Agilent Part Number	Final Concentration	XFe96 XF96 XFe24 (100 mL)	XF24[c] (100 mL)	XFp (10 mL)
Recommended Media[a, c]	Seahorse XF DMEM Medium, pH 7.4 / 103575-100 Seahorse XF RPMI Medium, pH 7.4 / 103576-100	-	99 mL	N.C.	9.9 mL
Alternative Media[b]	XF Base Medium (w/out Phenol Red) / 103335-100 XF RPMI (w/out Phenol Red) / 103336-100 XF Base Medium / 103334-100	-	99 mL	99 mL	9.9 mL
Required Supplements	Seahorse XF L-Glutamine (200 mM solution) / 103579-100	2 mM	1.0 mL	1.0 mL	100 µL

[a]XF DMEM and RPMI Medium, pH 7.4 have a pre-adjusted pH value and do not require adjustment of pH upon addition of XF supplements. See Method 1 below for preparation
[b]XF Base Medium (without Phenol Red), XF RPMI (without Phenol Red) or XF Base Medium may be substituted for XF DMEM or RPMI Media pH 7.4. If substituted, adjustment of final media pH value to 7.4 is required. See Method 2 below for preparation.
[c]Seahorse XF DMEM Medium pH 7.4 and RPMI Medium, pH 7.4 are not compatible with XF24 Analyzers.

Method 1: Using XF DMEM Medium pH 7.4 or XF RPMI Medium pH 7.4

Equipment Required:

- 37°C water bath

Agilent Seahorse XF DMEM Medium pH 7.4 and XF RPMI Medium pH 7.4 are designed to provide:

- Convenience: no adjustment of final pH is required when used as recommended with Agilent Seahorse XF Supplements.
- Consistency: Low concentrations of HEPES buffer (5 mM, DMEM; 1 mM, RPMI) provide more consistent XF data.
- Quantitation: Using assay medium with a fixed buffer capacity allows for quantitative measurement of proton efflux rate (PER).

1. Warm appropriate volume of XF DMEM Medium pH7.4 or XF RPMI Medium pH 7.4 to 37°C in a sterile bottle. In general, 100 mL is sufficient for one XFe96/XF96 *or* one XFe24/XF24 plate. 10 mL is sufficient for one XFp plate.
2. Add appropriate volumes of XF supplements (XF Glucose solution, XF Pyruvate solution and/or XF L-Glutamine solution) indicated in the table(s) above, based on assay type and XF platform used.
3. Incubate the final XF Assay Medium at 37°C until ready for use.

Method 2: Using XF Base Medium (without Phenol Red), XF RPMI Medium (without Phenol Red) or XF Base Medium

Equipment Required:

- 37°C water bath
- 0.2 µm Sterile Filter
- Calibrated pH meter
- 1 N NaOH

1. Warm appropriate volume XF Base Medium without Phenol Red, XF RPMI Medium without Phenol Red or XF Base Medium to 37°C. 100 mL is sufficient for one XFe96/XF96 or one XFe24/XF24 plate. 10 mL is sufficient for one XFp plate.
2. Add appropriate volumes of XF supplements (XF Glucose, XF Pyruvate and/or XF L-Glutamine solution) indicated in the table(s) above, based on assay type and XF platform used. Note that if performing the XF Real-Time ATP rate assay or the Glycolytic Rate Assay, XF HEPES buffer must be added *when using alternative media.*
3. Adjust pH value of the medium to 7.4 using 1 N NaOH. Note: pH value will change quickly upon addition of NaOH, use small volumes and add slowly to adjust pH value.
4. Sterilize assay medium with a 0.2 µm filter.
5. Incubate the Assay Medium at 37°C until ready for use.

Notes and Suggestions:

- Glucose, L-glutamine, and pyruvate are the most commonly added substrates to cell culture and XF assay medium. Oxidation and/or utilization of these substrates is required for XF assays. In XF assays, these substrates are typically provided in saturating concentrations (reflected in the tables above) to ensure that they are not limiting the rates of respiration (OCR) and/or extracellular acidification (ECAR).
- It is recommended that substrate concentrations of 10 mM glucose, 1 mM pyruvate and 2 mM L-glutamine are used in initial XF assays (except the Glycolysis Stress Test, 2 mM glutamine only). Note, however, that substrate requirements can be cell-type specific and may need to be determined empirically. Often, matching growth medium substrate concentrations is a good starting point.
- A consistent pH value of 7.4 +/- 0.2 for XF DMEM Medium pH 7.4 and XF RPMI Medium pH 7.4 is ensured when using Agilent Seahorse XF Supplements at the following final concentration ranges:

Agilent Seahorse XF Supplement	Range of final supplement concentrations recommended for final pH of 7.4 +/- 0.2
Seahorse XF Glucose (1.0 M solution)	0 – 10 mM
Seahorse XF Sodium Pyruvate (100 mM solution)	0 – 1 mM
Seahorse XF L-Glutamine (200 mM solution)	0 – 2 mM

- It is recommended that glutamine-alanine dipeptide (e.g. GlutaMAX) NOT be used as a glutamine source in XF assays.
- It is recommended to incubate assay medium at 37°C for no more than 4 hours, as substrates, such as glutamine, can degrade.
- For more information on XF assay media composition by cell type and/or assay type, please see: the cell reference data base: http://www.agilent.com/cell-reference-database and/or Seahorse XF Assay Guides and Templates: http://www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-assay-guides-templates.

www.agilent.com/chem

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